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FACTORS EFFECTING X-RAY INHIBITION OF ANTIBODY FORMATION

L. O. JACOBSON, M.D., AND M. J. ROBSON, B.S.
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THE fact that a single total-body exposure to x-rays inhibits antibody formation is wall documented.[1-5] It has also been demonstrated that the time of antigen injection relative to irradiation determines in a large measure the degree of inhibition.[1, 3, 6] Exposure of rabbits to dosages of total-body x-radiation ranging from 250 to 800 r effectively suppresses the development of appreciable hemolysin titer to intravenously injected sheep red cells when the latter are administered during the twenty-four hour period before or the forty-eight hour period after irradiation.[3-5]

It would appear from these various observations that irradiation either destroys the cells concerned in antibody formation or reduces their functional capacity to react in the normal way to the injected antigen. It is not known with certainty in which cells of the body this function normally resides, but it has been postulated in a broad sense that those belonging to the so-called reticulo-endothelial system are responsible. Others postulate a more definite cell, such as the plasma cell,[7-9] the lymphocyte,[10, 11] or the macrophage.[12, 13] Early experiments suggested that suppression of antibody formation by irradiation correlated well with the destructive effect of irradiation on the blood-forming tissue or more specifically with the effect of irradiation on the lymphatic tissue.[1, 2] Craddock and Lawrence[4] have reported that although a dose of 250 r total-body x-radiation would markedly inhibit antibody formation, ". . . this depressant effect could not be related entirely to the depression of lymphoid tissue." These authors came to this conclusion because the inhibition of lymphopoiesis was too incomplete to account for the drastically reduced response to the antigen if lymphocytes were specifically concerned with this function.

It was recently shown by Jacobson and associates[5] that if the spleen of the rabbit is shielded with lead during total-body exposure to 800 r, the capacity to form antibodies to an antigen injected twenty-four hours after irradiation is retained. Furthermore, it was found that the originally shielded spleen can be removed three days after the antigen injection without materially altering the capacity to form antibodies in animals exposed to radiation.

The spleen-shielding irradiation experiment referred to above[5] implied that either antibody formation was initiated in the shielded spleen and, once initiated,

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could be carried on elsewhere in the body or that some factor (probably a noncellular substance) was given off by the spleen which neutralized the inhibitory effect of x-radiation on antibody formation in cells of the body. At the time of the antigen injection (twenty-four hours after exposure to 800 r total-body x-radiation) the bone marrow and lymphatic structures of the body are largely reduced to a reticulum stroma, whereas the originally shielded spleen is histologically normal. In order to eliminate the spleen as the site of the initiation of antibody formation and to test the hypothesis that the spleen in some way effectively restored the functional capacity of cells of the body to react to an antigen and produce antibodies, an experiment was devised involving splenectomy twenty-four hours after the irradiation-spleen-shielding procedure and twenty-four hours before antigen injection. In other words, the antigen would be given at a time when the maximum destruction of the various hematopoietic tissues had been reached. Removal of the originally shielded spleen twenty-four hours before the antigen injection eliminated the spleen as a site of fixation of the antigen and initiation of the process of antibody formation. On the other hand, the spleen, while in the circulation, could introduce into the circulation the factor or factors that might make other cells in the irradiated animal capable of performing this function. Such a proposition is reasonable since it is known that leaving the shielded spleen of mice in the circulation for an hour after total-body exposure to 1,025 r is sufficient to overcome the lethal effects of this dose, presumably as a result of the release by the intact spleen of some factor that makes functional recovery of cells throughout the body possible in time to avert death of the animal.[14, 15] Furthermore, it had been demonstrated that a similar "therapeutic" effect can be obtained by transplanting a few milligrams of mouse spleen into the peritoneal cavity of mice within forty-eight hours after exposure to a lethal dose of x-radiation (1025 r).[14]

MATERIALS AND METHODS

Young adult rabbits (Swift's "snuffle-free"), weighing approximately 2 kg., were used in this study. The rabbits were divided into seven groups and the experiments carried out as indicated in Table I. The technique of lead shielding the spleen during exposure to total-body x-radiation has been given in detail in a previous publication.[5]

The anesthetic used when surgical procedures were employed was intravenous Nembutal (38 mg. per kilogram of total body weight). Groups I and II were not irradiated but were subjected to a surgical procedure, Group III animals were given 800 r or 500 r total-body x-radiation without spleen shielding. Group IV was given total-body x-radiation, and the spleen was exteriorized but not shielded during the irradiation. No subsequent splenectomy was carried out in this group. Group V received 800 r or 500 r total-body x-radiation; the spleen was exteriorized during exposure but not shielded; twenty-four hours after irradiation the spleen was removed surgically. Group VI received 800 r total-body x-radiation with spleen shielding and no subsequent splenectomy was performed. Group VII received 800 r or 500 r total-body x-radiation with spleen shielding followed twenty-four hours later by splenectomy.

The animals of all seven groups received intravenous injections of 1 ml. of a 2 per cent suspension of washed sheep cells forty-eight hours after irradiation or forty-eight hours after the beginning of the experiment. All splenectomies were performed twenty-four hours after exposure; the antigen was administered twenty-four hours after removal of the spleen. The same batch of antigen (sheep red cells) was used throughout these experiments. The antigen was injected into rabbits from all groups on the same day.

Blood specimens for antibody titer determination were obtained from all rabbits immediately before the beginning of the experiment and seven, ten, fourteen, twenty-one, and twenty-eight days after immunization. The hemolysin titers were determined by making serial dilutions of the rabbit sera (1:10, 1:20, 1:40, etc., to 1:5120) in 0.5 ml. amounts, adding 0.5 ml. of diluted lyophilized guinea pig complement (2 units) and 0.5 ml. of a 2 per cent suspension of washed sheep red cells, incubating one hour at 37° C., and refrigerating overnight. The hemolysin titrations on each series (those animals treated and immunized on the same day) were done at the same time to eliminate differences in complement or sheep cell suspension. The titer of each particular serum was the dilution that gave complete macroscopic hemolysis.

Histologic Studies.—Separate groups of animals were set up paralleling those in Groups I to VII. At least two animals from each group were sacrificed on the second through the twelfth days after irradiation for histologic study. Hematopoietic tissues including the bone marrow (right femur), thymus, lymph nodes at the root of the mesentery, spleen (if present), liver, Peyer's patches, and appendix were taken for study. The tissues were fixed in Zenker-Formol, embedded in 20 per cent nitrocellulose, sectioned at six or eight μ, and stained with hematoxylin-eosin-azure II. The histologic findings will be reported in detail in a subsequent paper dealing with further studies on the same general subject.

Dosimetry.—The X-rays were generated in a 250-kilovolt machine operating at 15 milliamperes, with a 1.0 millimeter copper filter and a 3.0 millimeter Bakelite filter. The half-value layer in copper of the filtered beam was 2.0 mm. The exposures were measured with a Victoreen condenser r-meter equipped with a 100-r chamber, and measurements were made in air at the position occupied by the center of the animal's body. The dose rate averaged 15.5 r per minute at 33 inches; a total dose of 800 r or 500 r was delivered, as indicated in Table I.

RESULTS

As shown in Table I, the control, nonirradiated rabbits in Groups I and II which were subjected to surgery exhibited the usual antibody response to the intravenously-injected sheep red cell antigen. The antibody titers attained in these two control groups, with one exception (No. 5), were significant although relatively low when compared to our previous experience with the response of rabbits given this antigen.[5]

In the control, irradiated groups (Groups III, IV, and V) which were subjected to sham shielding involving surgery or splenectomy or no surgical procedure, the inhibition of antibody production was quite consistent. In only one animal (No. 21) was a titer above 1:20 achieved during the early period after antigen injection. In animals No. 18, No. 19, and No. 27, however, titers above 1:20 were achieved only on the twenty-first and twenty-eighth days after antigen injection. The three animals in Group VI which had spleen shielding during exposure to 800 r and no subsequent splenectomy, all attained an antibody titer of 1:40 or higher. Actually, the results obtained in Group VI are consistent with the results reported previously[5] and inclusion of this group was intended only as a control for the experimental group described below.

Of the eight animals in Group VII which had spleen shielding during exposure to 500 r or 800 r total-body x-radiation, a splenectomy twenty-four hours after irradiation and antigen administered twenty-four hours later, only one (No. 39) failed to attain an antibody titer of 1:80 or higher.

TABLE I. THE EFFECT OF LEAD SHIELDING OF THE SURGICALLY MOBILIZED SPLEEN FOLLOWED BY SPLENECTOMY TWENTY-FOUR HOURS LATER ON ANTIBODY RESPONSE

GROUP	RABBIT NO.	PREPARATION	ANTIGEN	HEMOLYSIN TITER AT INTERVALS AFTER IMMUNIZATION					
				CONTROL	7 DAYS	10 DAYS	14 DAYS	21 DAYS	28 DAYS
I	(1)	Spleen exteriorization only	1 c.c. 2% sheep cells intravenously 48 hours after beginning of experiment	0	1,280	2,560	*	1,280	640
	(2)	Spleen exteriorization only		0	160	640	*	320	320
	(3)	Spleen exteriorization only		10	10	320	*	160	160
	(4)	Spleen exteriorization only		20	160	320	320	160	320
	(5)	Spleen exteriorization only		0	20	20	10	20	10
	(6)	Spleen exteriorization only		0	40	80	80	80	40
II	(7)	Splenectomy only 24 hours prior to antigen injection	1 c.c. 2% sheep cells intravenously 24 hours after splenectomy	0	640	640	*	640	160
	(8)	Splenectomy only 24 hours prior to antigen injection		0	40	160	160	80	80
III	(9)	800 r total-body irradiation only	1 c.c. 2% sheep cells intravenously 48 hours after x-ray	10	0	†	†	†	†
	(10)	800 r total-body irradiation only		0	0	†	†	†	†
	(11)	800 r total-body irradiation only		20	0	0	0	0	0
	(12)	800 r total-body irradiation only		10	0	10	10	40	40
	(13)	500 r total-body irradiation only	1 c.c. 2% sheep cells intravenously 48 hours after x-ray	0	0	0	0	0	0
IV	(14)	800 r total-body irradiation with spleen exteriorized but no shielding	1 c.c. 2% sheep cells intravenously 48 hours after x-ray	0	0	0	*	20	†
	(15)			20	0	0	0	0	0
	(16)			0	0	0	0	0	0
	(17)	Same as above except 500 r total-body irradiation	1 c.c. 2% sheep cells intravenously 48 hours after x-ray	20	0	0	0	0	0
V	(18)	800 r total-body irradiation with spleen exteriorized but no shielding followed by splenectomy 24 hours after X-ray	1 c.c. 2% sheep cells intravenously 48 hours after x-ray	20	10	0	20	80	160
	(19)			0	0	0	10	40	80
	(20)			10	0	†	†	†	†
	(21)			10	160	80	†	†	†
	(22)			0	0	0	0	10	10
	(23)			0	0	0	0	10	10
	(24)			0	0	†	†	†	†
	(25)	Same as above except 500 r total-body irradiation	1 c.c. 2% sheep cells intravenously 48 hours after x-ray	0	10	0	0	10	20
	(26)			0	0	0	0	0	0
	(27)			0	0	0	20	40	40
	(28)			0	0	0	†	†	†
	(29)			0	0	0	0	0	0
VI	(30)	800 r total-body irradiation with spleen shielding	1 c.c. 2% sheep cells intravenously 48 hours after x-ray	0	160	160	*	80	80
	(31)			20	640	2,560	5,120	2,560	1,280
	(32)			0	20	40	40	†	†
VII	(33)	800 r total-body irradiation with spleen shielding followed by splenectomy 24 hours after X-ray	1 c.c. 2% sheep cells intravenously 48 hours after x-ray	0	10	80	*	80	160
	(34)			0	20	80	160	320	320
	(35)			20	*	*	80	160	320
	(36)			10	160	320	320	320	160
	(37)			0	80	320	640	320	160
	(38)	Same as above except 500 r total-body irradiation	1 c.c. 2% sheep cells intravenously 48 hours after x-ray	0	20	80	320	320	160
	(39)			40	10	0	0	10	10
	(40)			10	20	40	80	160	80

* Data not obtained.

DISCUSSION

These results indicate that the capacity to form antibodies to an injected antigen is retained in rabbits given 800 r or 500 r total-body x-radiation if the spleen is shielded during irradiation, left intact in the circulation twenty-four hours after irradiation, and then removed surgically; the antigen is given twenty-four hours after splenectomy and forty-eight hours after the original irradiation. It is obvious that maintenance of the originally shielded spleen in the circulation for twenty-four hours after exposure of the animal to 800 r or 500 r was sufficient to restore, at least partially, the functional capacity of cells in the body to produce antibodies. It is admitted that the production of antibodies may involve more than one cell type. If more than one step, involving perhaps more than one cell type, is essential in the process which culminates in antibody formation, it does not necessarily follow that irradiation inhibits all of these steps.

During the twenty-four hour period after irradiation but prior to splenectomy, cells from the shielded spleen undoubtedly enter the general circulation. Even if the shielded spleen is not removed, however, the number of lymphocytes entering the circulation is not sufficient during the first nine days after irradiation to elevate significantly the lymphocyte count above that of rabbits exposed to 800 r without spleen shielding.[16, 20] Cells entering the circulation even in relatively small numbers could be of significance if these cells colonized in various parts of the body and initiated a rapid repopulation. The hematopoietic tissues repopulated on this basis might be able to carry on antibody formation. Another possibility is that if cells entering the general circulation implant in the body, these foci of cells might elaborate the factor responsible for reinstitution of the cellular function under discussion. It seems more probable, however, that a noncellular factor enters the general circulation from the spleen and restores the functional capacity of cells in the body to act upon the antigen and produce antibodies. There is supporting evidence for the concept that the factor is a product of cells within the shielded spleen or of cells which have entered the circulation rather than primarily a proliferative process of the cells which have entered the circulation.[14, 15, 17] Further studies to clarify this problem are in progress.

These findings described above do not reveal "the cell type" which is chiefly concerned in antibody formation, but they do clarify to some extent the approach one may take to elucidate further the general problem. For ten to twelve days or longer after exposure of rabbits to doses of 800 r X-radiation the hematopoietic tissues are usually described as atrophic.[18, 19] Even after such a dose, however, the reticulum of the lymphatic tissues and bone marrow and the free and fixed macrophages remain essentially "histologically" intact. After the latent period, which is characterized by atrophy as far as free cells such as lymphocytes and myelocytes are concerned, orderly regeneration of hematopoietic tissues begins. Regeneration occurs mainly as a heteroplastic process from reticular cells and to a minor degree homoplastically from the few free cells that survive the irradiation procedure. If, in rabbits exposed to 800 r

with spleen shielding, the spleen is left intact in the circulation, regeneration of the lymphatic structures is well under way by six days.[5] The effect on hematopoietic recovery of leaving the shielded spleen in the circulation for only twenty-four hours has as yet not been investigated. Even in the absence of this information it seems logical to assume that if, indeed, the reticulum, macrophages, and the few surviving "free" cells are concerned in antibody formation under the conditions described in this paper, then presumably they have been at least partially restored to normal insofar as this function (antibody formation) is concerned. It should be emphasized that the factor (or factors) coming from the shielded spleen under the various conditions described in this study may have a salutary effect on cells of the irradiated body other than those of the hematopoietic system.

SUMMARY AND CONCLUSIONS

Rabbits were exposed to dosages of 800 r or 500 r total-body x-radiation with spleen shielding. Twenty-four hours later the spleen was removed surgically. After another twenty-four hour period (forty-eight hours after irradiation) a particulate antigen (sheep red cells) was given intravenously. The capacity of these animals to form antibodies (anti-sheep cell hemolysin) was compared with various control groups given the same antigen at the same time relative to the irradiation as the group described above. Controls consisted of (1) animals not irradiated but which had surgical exteriorization of the spleen only, (2) animals that were not irradiated but were subjected to splenectomy, (3) animals that were irradiated without spleen shielding or other surgical manipulation, (4) animals exposed to radiation with spleen exteriorization, without shielding, but with subsequent splenectomy, (5) animals that were irradiated with spleen shielding but had no subsequent splenectomy.

The data presented show that the capacity to form antibodies to an injected antigen is retained in rabbits exposed to 800 r or 500 r total-body x-radiation if the spleen is shielded during irradiation, left intact in the circulation twenty-four hours after irradiation, and then removed surgically even though the antigen is given twenty-four hours after splenectomy and forty-eight hours after irradiation. The fact that these rabbits retain the capacity to form antibodies even though hematopoietic tissue in the body is atrophic and that control rabbits exposed to 800 r or 500 r do not retain this capacity is considered to be a result of the functional restoration of cells in the body (such as free and fixed macrophages and reticular cells) by a humoral (noncellular) substance entering the general circulation from the originally shielded spleen during the twenty-hour hours prior to splenectomy.

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SOME EFFECTS OF INTRAVENOUS FAT EMULSIONS ON HUMAN SUBJECTS

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INTRODUCTION

THE advantages of parenteral fat as a source of calories have been pointed out by numerous authors.[1-15] Recently, some investigators have made progress in the development of fat emulsions that appear satisfactory in animal studies.[7, 10, 14, 15] Some data on the clinical use of fat emulsions have been published, although further clinical evaluation is desirable. Holt and associates[1] gave small injections to sixteen infants with nutritional deficiency for two to seven days. Clark and Brunschwig[3] gave fat intravenously to one patient whose only source of nutrition was parenteral. Helfrick and Abelson[5] gave complete intravenous feedings including fat for five days to a marasmic child. Gorens and others[9] reported injecting eleven human beings with 300 to 500 ml. of fat emulsion for an average of nine days. Shafiroff and co-workers[12] gave injections of fat emulsion to seventy-six surgical patients, each of whom received an average of four infusions of one liter. Neptune and co-workers[13] have reported giving 15 per cent fat emulsion to nine seriously ill patients without untoward effects and with evidence of fat utilization. It is the purpose of this paper to present the preparation and trial on human subjects of four fat emulsions. Observations include the effects of single and repeated injections.

PROCEDURE

A. *Preparation of Fat Emulsions.*—Common to all the emulsions were the olive oil and 5 per cent dextrose in distilled water. The variable constituents were the emulsifying agents.

Emulsion No. 1—10 per cent fat with three emulsifying agents: Span 20 0.5 per cent,* Asolectin 0.4 per cent,† sodium cholate 0.1 per cent.

Emulsion No. 2—10 per cent fat with Span 40 0.5 per cent,‡ Asolectin 0.4 per cent, and sodium cholate 0.1 per cent.

Emulsion No. 3—10 per cent fat with 1 per cent cerebrosides.§

Emulsion No. 4—10 per cent fat with 1 per cent lecithin fraction.‖

The preparation of Emulsion No. 4 will be discussed in full, since it was the one used most extensively. The waxy lecithin was divided into small pieces and added to fresh commercially prepared olive oil, which was placed on a steam bath until the lecithin had dissolved in the oil. The oil-lecithin and dextrose-water (5 per cent dextrose in water) phases were mixed in a Waring Blendor to make a coarse emulsion, and quickly put through the Cherry-Burrell dairy homogenizer¶ (previously rinsed with pyrogen-free dis-

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*Sorbitan Monolaurate.

†Asolectin was kindly supplied by Associated Concentrates, Incorported, 57-01 32nd Avenue, Woodside, Long Island, New York.

‡Sorbitan Palmitate.

§This material was kindly supplied through the courtesy of Dr. John R. Mote of Armour and Company, Chicago, Ill.

‖This material was kindly supplied through the courtesy of Dr. Percy Julian of Glidden and Company, Chicago, Ill.

¶The authors wish to acknowledge indebtedness to the Cutter Laboratory, Berkeley, Calif., for the funds used to purchase the homogenizer.

tilled water) fifteen times at 3,500 pounds per square inch of pressure. The resulting emulsion was filtered through a Buchner funnel using No. 50 Whatman filter paper. The emulsion was bottled, and at twenty-four hour intervals autoclaved twice at 121° C. and ten pounds pressure for ten minutes. The bottles were sealed and stored at 4° C.

Particle size was determined in each batch by sampling from the top layer of one bottle and examining this microscopically with the aid of an ocular micrometer. It was considered suitable if its general particle size was 0.2 to 0.5 micron in diameter with relatively few globules as large as 1 to 2 micra, and none larger.

Cultures were taken on agar plates, and were always found to be sterile.

Bottles of 10 per cent fat emulsion containing 1 per cent cerebroside or 1 per cent lecithin were stored at room temperature, and others in the refrigerator for over one and one-half years. Gross separation of fat occurred in the former specimens, while in the latter ones only a few oil droplets could be seen on the surface. Microscopic examination of a specimen obtained from beneath the surface of the refrigerated samples revealed the particle size to be unchanged.

B. *Clinical Testing on Patients—Single Injections.*—Seventy-nine patients from the general surgical and medical wards were given a single infusion of one gram of fat per kilogram body weight in a four-hour period. Thirty-nine patients received Emulsion No. 3, and 40 received Emulsion No. 4. Before, during, and after the infusion the following observations were made: blood pressure, pulse, respiration, temperature, urinalysis, plasma studies, skin reactions, and careful inquiry concerning the patients' subjective state for the development of any new symptomatology.

C. *Clinical Testing on Patients—Repeated Injections.*—A few patients were given daily injections for two to four weeks. During a control period and during the time of injections, the following determinations were made: red cell count, hemoglobin, hematocrit, white cell count, plasma nonprotein nitrogen, total serum protein, cephalin flocculation, thymol turbidity, alkaline phosphatase, body weight, and in one case total cholesterol and prothrombin time.

TABLE I. DAILY INJECTION* OF 500 C.C. 10 PER CENT FAT EMULSION (CONTAINING SPAN 40, ASOLECTIN, AND SODIUM CHOLATE) FOR TWENTY-FOUR DAYS

	PATIENT C. S.							
Determination	Sept. 13	Oct. 15	Oct. 16	Oct. 20	Oct. 26	Nov. 5	Nov. 11	Nov. 12
R.B.C.	4.40	4.81		4.92	4.75	4.28		5.16
Hb.	91	88		96	93	96		98
Hematocrit	48	48		50	49	50		53
W.B.C.	7150	7300		6500	9850	8400		9350
N.P.N.	43	41		40	38	38		36
Total protein	6.0	6.4		7.4	6.3	6.7		7.0
Cephalin flocculation		0						0
Thymol turbidity		4.5						6.7
Weight	102	104		102	102	107		106
Urine	Daily determinations for albumin, sugar, bile, urobilinogen, acetone, blood, and microscopic—negative on all occasions.							

First of daily 500 c.c. fat injections

Last day of injection

*Given daily injections from October 16 to November 11 except on the following days: Oct. 22, Oct. 31, Nov. 2, Nov. 9.

Two patients with generalized arteriosclerosis and senility were permitted to eat at liberty throughout the study. Patient C. S. was given 500 ml. of Emulsion No. 2 daily for twenty-four days (see Table I). Patient J. H. was given 500 ml. of Emulsion No. 3 for twelve days and 1,000 ml. daily for the next twelve days (see Table II).

A third patient, E. C., suffered from regional enteritis and complaints of vomiting, diarrhea, cramping pains, and 40 pounds weight loss. Only one set of control studies was

TABLE II. DAILY INJECTION* OF 10 PER CENT FAT EMULSION (CONTAINING 1 PER CENT CEREBROSIDE) FOR TWENTY-FOUR DAYS

	500 C.C. FOR TWELVE DAYS 1,000 C.C. FOR TWELVE DAYS PATIENT J. H.								
Determination	Sept. 13	Oct. 15	Nov. 3	Nov. 8	Nov. 15	Nov. 15	Nov. 22	Nov. 27	Nov. 29
R.B.C.	4.35	4.30		5.02	5.00		5.47		5.19
Hb.	90	80		90	104		100		95
Hematocrit	48	43		48	54		51		50
W.B.C.	10,450	5800		7050	7100		6050		6450
N.P.N.	38	41		43	40		36		43
Total protein	6.5	6.3		7.2	7.6		6.7		7.1
Cephalin flocculation	0			0			0		2+
Thymol turbidity	2.8			2.4			2.8		3.8
Alkaline phosphatase	2.3				3.0		1.9		4.4
Weight	104	102		106.5	105		105		105
Urine	Daily determinations for albumin, sugar, acetone, bile, urobilinogen, blood, and microscopic—negative on all occasions.								

First of daily 500 c.c. fat injections

First of daily 1,000 c.c. fat injections

Last day of injection

*Given daily injections from Nov. 3 to Nov. 27, with exception of Nov. 21.

obtained (at this time he was allowed all he wished per os). Complete parenteral feeding was begun and continued for 11 days. He received 1000 ml. 20 per cent fat emulsion containing 1 per cent lecithin as the emulsifying agent, 2000 ml. 5 per cent amino acids containing 5 per cent dextrose, and 600 ml. of Ringer's solution to which had been added 100 mg. vitamin B_1, 100 mg. vitamin C, 4.8 mg. vitamin K, and 10 ml. of 5 per cent calcium gluconate.

Three other patients who had lost weight were observed for a two-week control period during which time they were given weighed diets and 1000 ml. of 5 per cent dextrose intravenously daily. During this time the laboratory studies enumerated previously were made and repeated. In addition, bleeding time, platelet count, and daily weights were determined. When the latter were at a constant level, daily injections of one liter of 20 per cent fat emulsified with 1 per cent lecithin in 5 per cent glucose were substituted for the dextrose injections.

RESULTS

Emulsion No. 1 was discarded after preliminary tests indicated that it produced a phlebitis in human subjects. This effect was found to be due to Span 20.

Emulsion No. 2 did not cause thrombophlebitis, but inflammation resulted whenever the solution entered the subcutaneous tissue.

Emulsions No. 3 and No. 4 were given as single injections to seventy-nine patients without serious complication but with some reactions (see Fig. 1). Only minor variations in blood pressure were observed, there being an average rise of 5 to 10 mm. Hg during the injection. The pulse rate remained unchanged except in those who developed a febrile response and in these it increased comparably to the temperature. Examination of the urine for acetone, albumin, sugar, blood, and microscopically for cells or fat globules before and after the injection failed to reveal any changes in the postinfusion specimen. Plasma was studied microscopically before, during, and after injection with fat emulsion; an increase in the number of particles was seen, but no increase

in their size. Temperature rise proved to be the most commonly experienced reaction following the injection of the emulsion, occurring in one-third of the patients. The elevation began near the injection, quickly reached a peak, and within four to eight hours returned to normal, thus following a more or less typical pyrogen reaction. Seventeen persons who experienced a temperature rise of one degree or more developed a chill. Anorexia, nausea, vomiting, diarrhea, and headache occurred in some of the patients who developed a febrile response. A few patients developed lumbosacral pain, a feeling of substernal constriction, and shortness of breath in the first minute or two of the infusion,

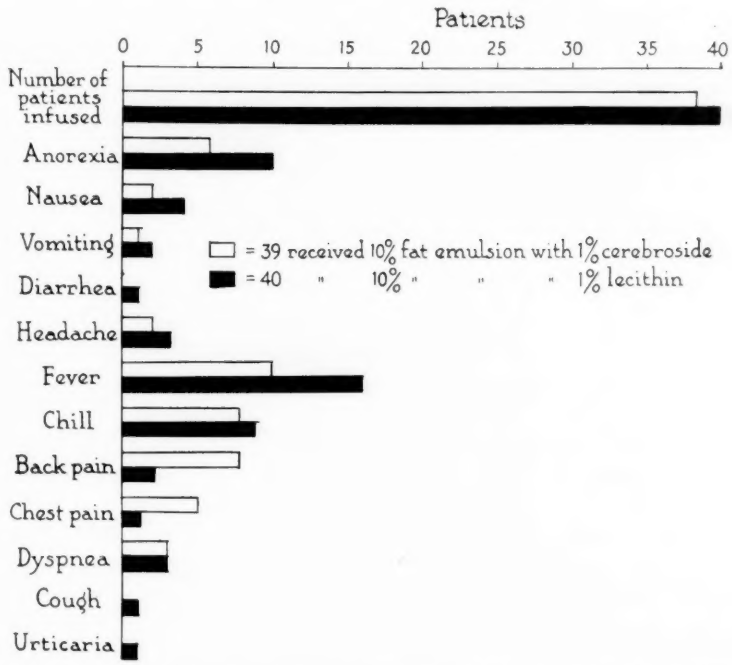

Fig. 1.—Frequency of symptoms produced by parenteral fat injections in seventy-nine human subjects.

which gradually disappeared in ten to twenty minutes, even though the injections were continued. These symptoms were more common in those receiving Emulsion No. 3, and it was found that a mixture of all the elements of Emulsion No. 3 with exclusion of the olive oil would produce the same reaction. The rate of injection influenced the tendency for symptoms to develop or recur. Persistent cough during the period of injection of Emulsion No. 4 was noted in one patient, and in another there developed a generalized urticaria. The cough disappeared spontaneously within three hours, and the urticaria was gone by the following day, the patient having received epinephrine and pyribenzamine.

Patient C. S. received Emulsion No. 2 for twenty-four days, and demonstrated a weight gain of 4 pounds in this time. There was essentially no change in the laboratory data (see Table I).

Patient J. H., who received Emulsion No. 3 for twenty-four days, demonstrated a weight gain of 3 pounds, and a definite increase in red cell count, hemoglobin, and hematocrit. All other determinations were essentially unchanged (see Table II).

During the eleven days patient E. C. received complete parenteral feedings, he improved subjectively and gained 9 pounds. The night following the eleventh day's injection, the patient vomited material which was benzidine positive and passed bright red blood per rectum. Fat injections were discontinued at this time. Bleeding time, clot retraction, platelet count and capillary fragility tests were found to be altered and were followed until normal (see Table III).

TABLE III. DAILY INJECTION OF 1,000 C.C. 20 PER CENT FAT EMULSION (CONTAINING 1 PER CENT LECITHIN "P") FOR ELEVEN DAYS

	PATIENT E. C.						
Determination	3-25	3-27	4-1	4-7	4-8	4-10	4-13
R.B.C.	2.45		3.20		2.85		
Hb.	37		55		50		
Hematocrit	25		29		24		
W.B.C.	4900		6200		11,000		
N.P.N.	52		50		30		
Total protein	6.3		5.8		5.4		
Cephalin flocculation	0		0		0		
Thymol turbidity	3.5		4.3		4.8		
Alkaline phosphatase	3.1		2.5		4.9		
Total cholesterol	140		160		160		
Prothrombin time	12 sec. Con-12		13 sec. Con-12		14 sec. Con-12		
Weight	110		119		119		
Bleeding time (min.)					5.5	22	0.5
Clot retraction (in 24 hours)					None	None	Normal
Platelet count					30,000	44,000	114,000
Capillary fragility (Rumpel Leed's)					Pos.	Pos.	Neg.

First of daily 1,000 c.c. injections 20% fat

Last day of injections

Blood transfusions were given the following days: March 26—1,000 c.c.; March 30—500 c.c.; March 31—500 c.c.; April 6—500 c.c.

A thrombocytopenia developed in two of the other three patients injected daily with one liter of 20 per cent fat emulsion. In one patient, the platelet count decreased from 225,000 to 5,000, and the bleeding time increased to six minutes after the third injection. The platelet count of another patient dropped from 305,000 to 20,000 after the second day of fat injections. The two fat injections given the third patient were without ill effect as judged by the tests performed.

DISCUSSION

Reports in the literature[8, 9, 12] indicate that the most common reactions to the injection of fat emulsion include fever, chills, nausea and vomiting, headache, and mild anemia. The febrile response has continued to be one of the most common reactions. Shafiroff and co-workers[12] attributed the fever to a

"lipemic plethora." Gorens and others[9] likewise found the febrile response to be a prominent problem. Neptune and associates[13] believe the febrile reactions are due to pyrogens that may be derived from commercial oils. Of the seventy-nine patient injections reported here, almost one-third developed a febrile response, and some of these a chill typical of a pyrogen reaction.

Investigations on the nature of pyrogens[16, 17] indicate that they are extremely soluble polysaccharides derived from the growth and disintegration of bacteria. Studies made to determine the origin of the pyrogenic reactions will not be presented in detail. However, it was found that freshly prepared fat emulsions may not give pyrogen reactions* immediately after preparation, but may do so after a few weeks, regardless of storage temperature and despite the fact that they remain sterile with no change in particle size. Studies on solutions of emulsifying agents indicate that these substances were the most probable source of pyrogens.

The development of back and chest pain and dyspnea during fat injection are similar in character to that described by other investigators[18] as a nitritoid reaction.

Cough and urticaria were considered allergic phenomena by virtue of the prompt relief to administration of epinephrine and pyribenzamine. Similar reactions have been reported to occur during the use of another emulsion with different constituents.[12]

Investigators have reported the development of a secondary anemia in experimental animals receiving fat injections over a prolonged period of time. Collins and associates[19] injected a number of dogs with solutions containing varying amounts of fat and a constant amount of emulsifying agents with resulting decrease in hemoglobin and hematocrit. Meng and Freeman[7] found that the injection of an emulsion containing Asolectin, Span 20, and sodium cholate for emulsifying agents over a prolonged period in dogs resulted in a decrease in red cell count, hemoglobin, and hematocrit. Lerner, Chaikoff, and Entenman[10] found a similar decrease in dogs receiving emulsions containing glycerol monostearate as the stabilizer. Clark and Brunschwig[3] noted a slight decrease in hemoglobin in a patient who was fed parenterally for seventeen days including injections of fat emulsion on eight of these days. Mann and associates[8] reported that giving fat emulsion to two patients for three and eight days, respectively, was associated with some decrease in the hemoglobin and hematocrit. Of the two patients reported here who received daily fat injections for twenty-four days, one evidenced increased hemoglobin and hematocrit but slightly lowered red cell count, while the other demonstrated a decided increase in all three. The other patients who received 20 per cent fat with 1 per cent lecithin manifested hematologic changes previously described. The thrombocytopenia that developed was probably due to increased intake of parenteral fat. It is not possible at this time to state whether this was due to suppression in formation or to increased destruction of thrombocytes.

*Pyrogen testing in animals was performed by Abbott Laboratories through the courtesy of Dr. D. V. Frost.

There was no evidence of gross renal damage, since the nonprotein nitrogen remained within normal range during the periods of injection, and there were no cells, casts, or albumin in the urine.

There was no evidence of impairment in liver function in any of these patients as reflected in the total protein, cephalin flocculation, thymol turbidity, alkaline phosphatase, or presence of bile or urobilinogen in the urine. No comparable information on human subjects has been reported, but in the experimental animal two investigators noted a slight reduction in the excretion of rose bengal,[15, 20] while others found normal excretions (one using rose bengal[7] and the other bromsulfalein[14]). McKibben and co-workers[20] found an accumulation of fat in the reticuloendothelial cells in the livers of dogs receiving fat emulsion, but none in one dog who received only the emulsifying agent. Mann and collaborators[8] have reported five post-mortem examinations on human beings who had received fat injections, and in none was there found any abnormality due to the fat injections.

A comparison of the histologic findings in rats and dogs injected with a fat emulsion or fatty chyle has been reported recently by Murray and Freeman,[21] who found some differences in the distribution of fat ten minutes after injection.

SUMMARY

1. The preparation, stability, pyrogenicity, and effects produced by four fat emulsions have been observed in relation to their administration to seventy-nine human subjects.

2. Daily injections of a 10 per cent fat emulsion (one gram fat per kilogram body weight) for twenty-four days in two human subjects produced no evidence of toxicity.

3. Daily injections of 20 per cent fat emulsions (3 to 4 Gm. fat per kilogram body weight) given from one to eleven days to four patients was associated with a decreased platelet count and prolonged bleeding time.

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HEPATIC FUNCTION AND CHOLESTEROL TOLERANCE IN THE DOG

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THE reduced tolerance for cholesterol demonstrated[1, 2] in protein deficient dogs may have resulted from impaired hepatic function or from some other effect of protein deficiency. To further clarify this question it seemed desirable to use a more specific means of impairing hepatic function and to determine the effects of cholesterol ingestion under this circumstance. For this purpose chronic liver insufficiency was produced by Eck fistula formation,[3] which deprives the liver of its portal blood supply. A moderate degree of atrophy and some fatty metamorphosis usually results[4] from this reduction in circulation. Some of the hepatic functions impaired by this operation have been enumerated elsewhere.[5]

PROCEDURE

Healthy, adult mongrel dogs of both sexes maintained on a stock diet of bread, milk, Karo syrup, and yeast were used for this study. The daily diet contained approximately 178 Gm. of carbohydrate, 24 Gm. of fat, and 42 Gm. of protein. Wilson's crystalline cholesterol dissolved in corn oil (10 per cent solution) was heated daily to the point of solution and given by stomach tube. The diet and supplemental feeding of cholesterol in oil (0.3 Gm. per kilogram) or of oil (3 ml. per kilogram) were given six days per week. The various groups included in the study and the duration of control and experimental periods are indicated in Table II.

Liver function was followed every two weeks by rose bengal dye clearance, serum alkaline phosphatase[6, 7] and by prothrombin times[8] at less frequent intervals. Plasma lipids were also estimated every two weeks on an extract of fasting heparinized plasma prepared according to Bloor's method.[9] Lipid phosphorus was estimated according to the method of Youngburg,[10] using Gomori's method[11] for phosphorus estimation and a factor of 25 for conversion to phospholipid. Total plasma fatty acids were determined according to the method of Smith and Kik[12] with minor modifications. Neutral fat was calculated according to a formula given by Thannhauser.[13] The method for free and total cholesterol of plasma was Clarke and Marney's modification[14] of the Schoenheimer-Sperry method.

Upon completion of the experiment the animals were anesthetized with nembutal and exsanguinated. A complete autopsy was performed and samples of liver tissue were taken for analysis. They were extracted according to the procedure of Best, Channon, and Ridout.[15] The total lipid content of the liver was taken as the ethyl ether soluble fraction of the alcohol-ether extract. Aliquots of the ether extract were used for determination of various fat fractions by the methods used on plasma. The content of cholesterol esters in liver tissue was expressed as cholesterol oleate, obtained by multiplying the bound cholesterol by 1.68.[16] Tissue phospholipids were calculated by multiplying the lipid phosphorus by the factor 22.7.[17]

RESULTS

The reduction in liver function following the Eck fistula operation was similar to that reported elsewhere.[5] The prothrombin times in the Eck fistula dogs averaged about 55 per cent of normal and were not appreciably changed

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TABLE I. EFFECT OF CHOLESTEROL AND ECK FISTULA ON PLASMA ON LIPIDS*

	NORMAL DOGS				ECK DOGS			
	CONTROL	OIL	CONTROL	OIL + CHOLESTEROL	CONTROL	OIL	CONTROL	OIL + CHOLESTEROL
Total cholesterol	208	213	223	255	90	115	119	386
Free cholesterol	48	55	56	67	24	31	33	150
Ester cholesterol	160	158	167	188	66	84	86	236
Phospholipids	422	413	431	415	232	260	245	371
Neutral fat	21	59	42	79	95	45	4	120

*All values on experimental diets are for the twenty-fourth week except those for normal dogs receiving oil, which are for the sixteenth week.

after the supplemental feedings were given. Cholesterol in oil feedings to the Eck fistula dogs caused a further decrease in the dye clearance and an elevation of the alkaline phosphatase. Similar feedings to the normal dogs caused some reduction in the dye clearance with only a slight elevation in the alkaline phosphatase. In the two oil-fed Eck fistula dogs there was a decrease in the dye clearance and no change in the alkaline phosphatase, while in the two oil-fed normal dogs the liver function tests remained the same.

The average plasma lipid values of the Eck fistula dogs were definitely lower than for the normal animal (Table I). Cholesterol in oil fed to Eck fistula dogs caused an increase in the fasting plasma lipid levels over control values for the group, while the other groups showed only a slight increase from the supplemental feedings after twenty-four weeks (Table I). The cholesterol-fed Eck fistula dogs showed a gradual increase in plasma total cholesterol to maximum values ranging between 400 and 724 mg. per cent, the average increase being 248 mg. per cent. The average increase in plasma

TABLE II. AVERAGE AND RANGE VALUES OF PLASMA TOTAL CHOLESTEROL

		CONTROL PERIOD			EXPERIMENTAL PERIOD		
DOG GROUPS	NO. DOGS	WEEKS	RANGE (MG. PER CENT)	AVERAGE (MG. PER CENT)	WEEKS	RANGE (MG. PER CENT)	AVERAGE (MG. PER CENT)
Normal dogs receiving oil feedings	2	2	145-232	180	16	201-280	234
Normal dogs receiving cholesterol in oil feedings	2	11	170-266	212	24	242-391	320
Eck dogs receiving only basic diet	3	19-40	70-158	97			
Eck dogs receiving oil feedings	2	4-10	60-120	93	37-42	72-190	123
Eck dogs receiving cholesterol in oil fedings	5	7-11	77-175	122	24-37	198-724	370

		CONTROL PERIOD			EXPERIMENTAL PERIOD					
					NORMAL			ECK FISTULA		
DOG GROUP	NO. DOGS	WEEKS	RANGE (MG. PER CENT)	AV. (MG. PER CENT)	WEEKS	RANGE (MG. PER CENT)	AV. (MG. PER CENT)	WEEKS	RANGE (MG. PER CENT)	AV. (MG. PER CENT)
Cholesterol-fed normal dogs in which Eck fistula operation was done	2	6	136-260	180	12	202-346	272	12	270-408	358

total cholesterol when cholesterol was fed to two normal dogs was 108 mg. per cent (see Table II). It should be pointed out that the initial plasma cholesterol values for the Eck fistula dogs were lower than for the normal animals. Only a slight elevation in plasma total cholesterol occurred in normal and Eck fistula dogs that received oil in addition to the basic diet (Table II).

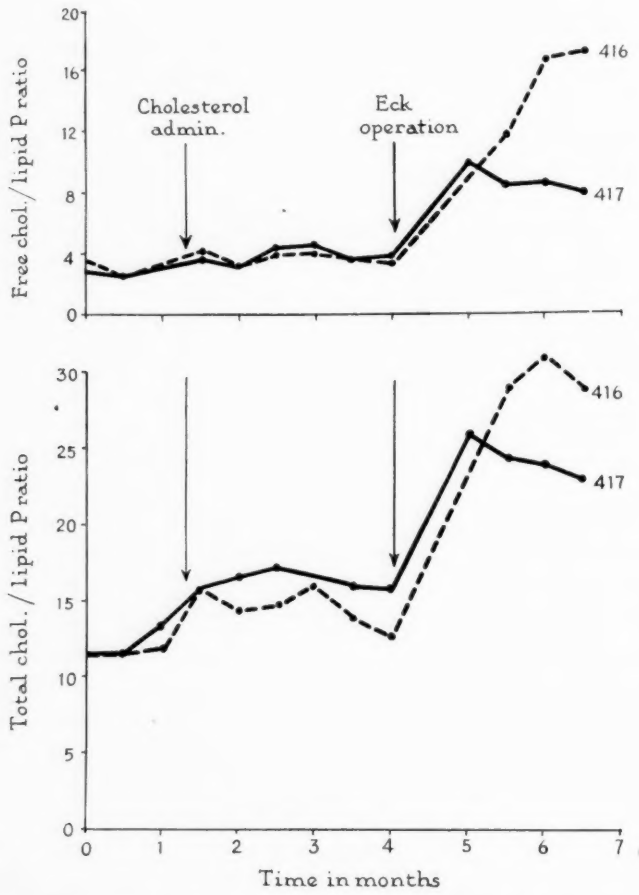

Fig. 1.—Demonstrating the effect of Eck fistula formation on the ratio of total and free cholesterol to lipid phosphorus in the serum and associated changes in dye clearance and serum alkaline phosphatase.

After twenty-four weeks of cholesterol feeding, the percentage of esterified cholesterol in the plasma of Eck fistula dogs was 61 per cent (S.D. 4.8), while in the control animals the average was 75 per cent (S.D. 2.9).

The plasma phospholipids of the cholesterol-fed Eck fistula dogs rose above their initial values, but the increase was not in proportion to the rise in total and free cholesterol. In contrast, the plasma phospholipids of the cholesterol-fed normal dogs rose at a rate that more nearly paralleled the rise of the total and the free cholesterol. The average total cholesterol/lipid

phosphorus ratio was increased in four cholesterol-fed Eck fistula dogs from the control value of 11.5 to 25 at the end of the twenty-fourth week of the feeding period, whereas in two cholesterol-fed normal dogs it was increased from the control value of 12.5 to 15.3. Fig. 1 shows the slight rise in the ratios upon feeding cholesterol to two normal dogs and how the ratios were markedly elevated after the Eck fistula operation had altered hepatic function (Fig. 2) in these two dogs while the feedings were continued. The ratios were elevated because the total and free cholesterol fraction rose after the operation, whereas the lipid phosphorus decreased from the preoperative average of 18 mg. per cent to a postoperative average of 15 mg. per cent.

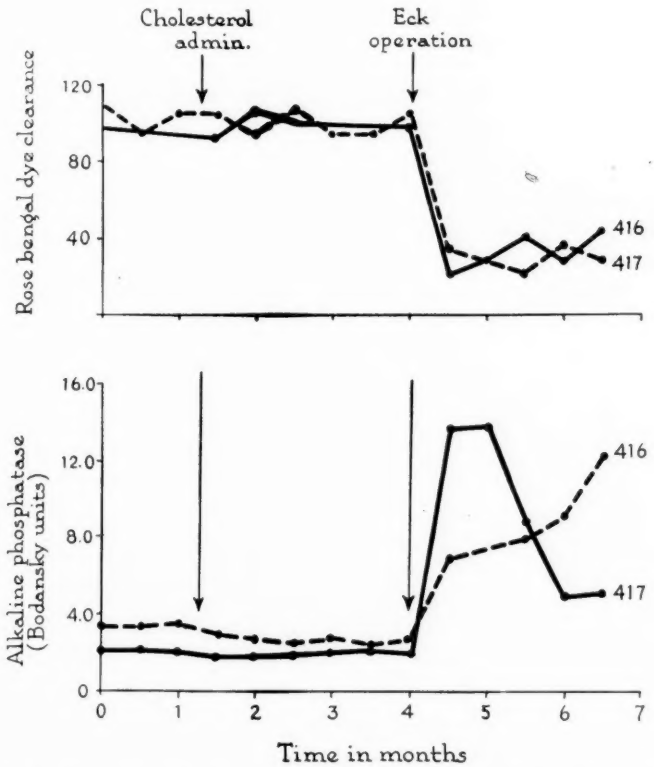

Fig. 2.—Demonstrating the effect of Eck fistula formation on the ratio of total and free cholesterol to lipid phosphorus in the serum and associated changes in dye clearance and serum alkaline phosphatase.

In Fig. 3 the lipid phosphorus and total cholesterol values for all the different groups of dogs have been plotted against each other. The first straight line shows the linear relationship between lipid phosphorus and total cholesterol for normal and Eck fistula dogs, with or without oil feedings. The factors for the slope and the intercept of this line were derived by the method of least squares.[18] In the cholesterol-fed normal dogs (line 2) the proportions between the lipid phosphorus and total cholesterol was slightly changed from

TABLE III. AVERAGE AND RANGE VALUES OF LIVER LIPID ANALYSIS

DOG GROUPS	NO. OF DOGS	AVERAGE TIME AS ECK DOG (WEEKS)	WEEKS ON OIL OR CHOLESTEROL	TOTAL LIPID WET LIVER (PER CENT)		FREE CHOL. WET LIVER (PER CENT)		TOTAL CHOL. WET LIVER (PER CENT)		ESTER* CHOL. WET LIVER (PER CENT)		PHOSPHOLIPID WET LIVER (PER CENT)		NEUTRAL FAT WET LIVER (PER CENT)	
				RANGE	AV.	RANGE	AV.	RANGE	AV.	RANGE	AV.	RANGE	AV.	RANGE	AV.
Normal dogs receiving oil	2		16	5.2-5.3	5.2	0.14-0.16	0.15	0.22-0.23	0.22	0.11-0.14	0.12	2.5	2.5	0.4	0.4
Normal dogs receiving cholesterol in in oil	2		24	9.0-11.8	10.4	0.25-0.28	0.26	1.14-0.60	0.87	0.57-1.44	1.0	2.7-3.9	3.3	0.8-5.5	3.1
Eck dogs receiving only basic diet	3	44		6.2-16.7	12.5	0.14-0.21	0.18	0.24-0.36	0.32	0.05-0.36	0.22	2.2-2.3	2.2	0.9-10.3	6.4
Eck dogs receiving oil	2	48	37-42	7.5-13.4	10.4	0.22-0.24	0.23	0.28-0.46	0.37	0.10-0.36	0.23	2.4-3.4	2.9	1.4-8.4	4.9
Eck dogs receiving cholesterol in oil	6	38	24-37	28.8-36.9	33.9	0.27-0.67	0.41	2.5-10.5	6.9	3.6-16.4	10.9	1.7-2.1	1.9	13.0-28.0	18.0

*Ester cholesterol has been expressed as cholesterol oleate.

that of the normal dogs, and in the cholesterol-fed Eck fistula dogs (line 3) it was markedly altered. On plotting all the lipid phosphorus values against the free cholesterol values a similar relationship was found to that shown in Fig. 3.

The neutral fat level was not appreciably elevated in the cholesterol-fed Eck fistula dogs as compared to the controls (Table I). In a particular animal the neutral fat would sometimes vary from 200 mg. per cent to zero on successive determinations. It is to be recalled that the neutral fat fraction was calculated by difference from the values of the other lipid components.

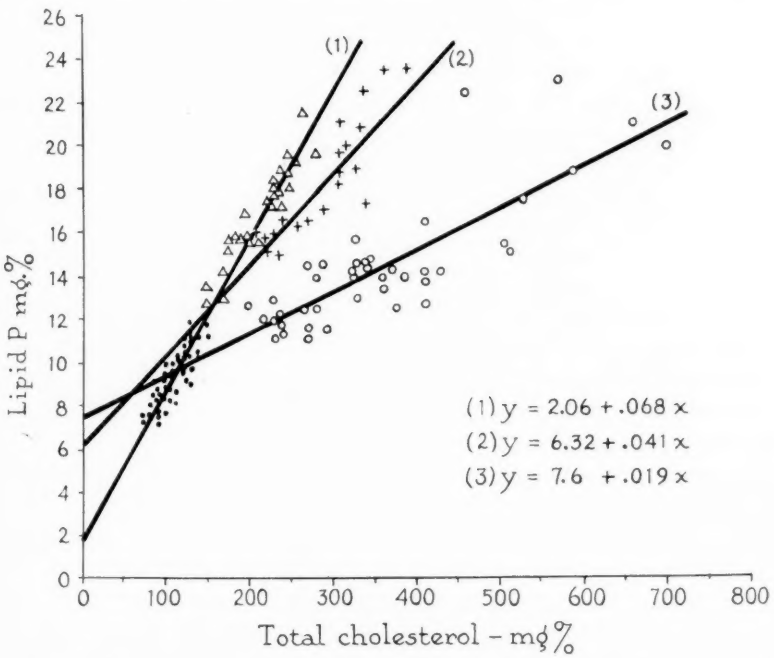

Fig. 3.—Demonstrating the effect of cholesterol feeding upon the relation of lipid phosphorus to total cholesterol in the serum of normal and Eck fistula dog.

(1) △△△△ Normal dogs with and without oil feedings.
(1) ●●●● Eck dogs with and without oil feedings.
(2) + + + + Normal dogs receiving cholesterol in oil feedings.
(3) ○○○○ Eck dogs receiving cholesterol in oil feedings.

Cholesterol in oil feedings to Eck fistula dogs resulted in very fatty livers as shown by chemical analysis (Table III). The fat was made up primarily of neutral fat and cholesterol esters. In the cholesterol-fed normal dogs there was only a slight increase in the content of the liver lipids. In the Eck fistula dogs receiving only the basic diet the amount of liver fat varied from a normal amount to a moderate increase. Oil feedings to the Eck fistula dogs and to the normal dogs did not increase the lipid content of the liver. Microscopic sections of the livers confirmed the findings of the lipid analyses. In addition a mild amount of diffuse hepatic fibrosis was noted in two of the cholesterol-fed

Eck fistula dogs, one of which had received the feedings for 24 weeks and the other for 46 weeks. In two of the control Eck fistula dogs receiving only the basic diet there seemed to be some increase in periportal fibrous tissue.

Renal and bladder stones were found in four of the five control Eck fistula dogs and in one of the seven Eck fistula dogs receiving cholesterol feedings. Two of the dogs that had stones were Dalmatians; their calculi were of the stag horn type. Roentgenograms of these stones showed them to be laminated with nonopaque central nuclei surrounded by layers of opaque material. It was determined by analysis that these stones largely consisted of uric acid and ammonium magnesium phosphate; only relatively small amounts of calcium were present.

Peptic ulcers were found in four of the seven Eck fistula dogs receiving cholesterol and in none of the five control Eck fistula dogs. In one of these the ulcer bled so much that the dog was sacrificed, and in another the dog died because the ulcer perforated.

Macroscopic or microscopic evidence of atherosclerosis was not found in any of the dogs.

DISCUSSION

The liver may influence the rate of cholesterol absorption, synthesis, destruction, excretion, conversion to specific steroids, and deposition in the tissues. Although bile does contain free cholesterol, the amount excreted by this route varies little with the blood cholesterol level and the dietary intake of cholesterol.[19] Arhens and Kunkel[20] have noted a rough correlation between the serum bilirubin level and the elevation of serum lipid levels in the hypercholesterolemia associated with intrahepatic obstructive jaundice. In the present experiment jaundice and hyperbilirubinemia were not present, except in two dogs in the last few weeks of life. Cholesterol destruction has been suspected of occurring in the liver, but as Thannhauser stated in 1947,[13] actual proof has never been established. Definite proof of cholesterol destruction by the body is indicated by the finding reported by Gould[21] that an appreciable fraction of C_{14} was found in respiratory carbon dioxide after giving cholesterol labeled with C_{14} to rats and mice. In the present experiment the most likely explanation for the elevated plasma cholesterol level in cholesterol-fed Eck fistula animals would seem to be that the liver with decreased function was unable to catabolize the exogenous cholesterol as rapidly as the normal liver.

Popjak[22] found that in cholesterol-fed rabbits and from lipid data in the literature that there was a relationship between the plasma lipid phosphorus and the free cholesterol. When the values were plotted against each other the points approximated a smooth curve, or were linear if their common logarithms were plotted against each other. The lipid phosphorus and total cholesterol were related only up to cholesterol values of 600 to 800 mg. per cent. Recently Albrink, Man, and Peters[23] have presented data on human subjects indicating that the lipid phosphorus and free cholesterol are related.

In the present experiment the ratio of free and total cholesterol to lipid phosphorus was definitely increased in the cholesterol-fed Eck fistula dog, as compared to the control animals. This disturbed relation was caused by higher plasma levels of cholesterol and relatively lower levels of lipid phosphorus in the cholesterol-fed Eck fistula dogs. The decreased level of phospholipid probably reflects the reduced capacity of the liver to synthesize these compounds. Others have shown[24, 25] that the liver is the principal source of plasma phospholipids. It has been reported that atherosclerosis develops in the rabbit when hypercholesterolemia is associated with an elevated cholesterol/phospholipid ratio, but does not occur if this ratio remains in the normal range.[26, 27] However, no atherosclerotic changes were detected in the animals included in this study despite the altered cholesterol/phospholipid ratios in the cholestreol-fed Eck fistula animals. Perhaps the duration of the study was too short to demonstrate this effect.

Since the livers of the cholesterol-fed Eck fistula dogs became very fatty in contrast to the slight amount of fat infiltration in the livers of the cholesterol-fed normal dogs, it appears that the Eck liver with impaired function has a decreased ability to dispose of exogenous cholesterol resulting in a marked accumulation of cholesterol esters and neutral fat within its substance. This finding, as well as the hypercholesterolemia of the cholesterol-fed Eck fistula dog, indicates that hepatic function plays an important role in the disposal of ingested cholesterol.

SUMMARY

The following differences were demonstrated between normal and Eck fistula dogs fed cholesterol in oil: (a) A greater increase in plasma cholesterol was observed in cholesterol-fed Eck fistula dogs. (b) There was a more marked increase in the plasma cholesterol/phospholipid ratio in the Eck fistula animal than in the normal animal fed cholesterol. (c) A much greater increase in the fat content of the liver occurred in the Eck fistula dog than in the normal animal fed cholesterol.

It is thought that the accumulation of cholesterol in blood and liver results from the inability of the Eck liver to catabolize this substance as well as can the normal liver.

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ELECTROPHORETIC ANALYSIS OF SERUM IN A CASE OF MULTIPLE MYELOMA ASSOCIATED WITH AMYLOIDOSIS

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SINCE the original study of Longsworth, Shedlovsky, and MacInnes, a number of publications have dealt with the electrophoretic analysis of the proteins in multiple myeloma.[1-11] This research demonstrated the value of the electrophoretic pattern in diagnosis of this disease.

In general, three types of multiple myeloma can be distinguished by electrophoretic methods. The first shows an increase in beta globulins, the second in gamma globulins. A third type shows components of variable size migrating between the gamma and beta types, which Gutman and his group[4-5] called the "M" component, and others[3, 12, 13] beta 2 or gamma 1. This component can be distinguished readily from fibrinogen.

The present paper describes the alterations in the serum proteins in a case of multiple myeloma associated with amyloidosis. A few electrophoretic analyses have been performed on body fluids in amyloid disease. Luetscher[15] has investigated the serum and urine of a patient with amyloidosis with chronic tuberculosis. Others[14] have studied renal amyloidosis. However, to our knowledge no comprehensive investigation of the characteristic changes in the electrophoretic patterns in various diseases associated with amyloidosis has been carried out.

METHOD

Serum from fasting morning blood samples was diluted with one to three parts of buffer, depending on the protein concentration of the serum. Sodium citrate-veronal buffer (pH 8.6 at 25° C., μ 0.1) of Longsworth, as modified by Deutsch and Goodloe[16] was used. In some tests the sodium veronal-veronal (barbital) buffer (pH 8.6; μ 0.1) of Longsworth[17] was used. Controls of normal human serum were run with both buffers. The serum was dialyzed for two hours at room temperature by the method of Reiner and Fenichel[18] with one change of buffer at the end of the first hour. In two experiments, using the barbital buffer, the buffer solution outside the cellophane bag was agitated during dialysis by a magnetic stirrer† for twelve to fifty-two hours at 7° C. This procedure was considered to involve less risk of protein denaturation. A mircrocell of 2 ml. capacity previously described by Ross and associates[19] and Cohen and co-workers[20] was used, and the electrophoretic technique followed was that of Longsworth.[17] During experiments using the veronal-sodium citrate buffer a field strength of about 9 volts per centimeter, and a current of 7-8 milliamperes was maintained

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†Magnetic stirrer constructed by Mr. R. Schaeffer, Department of Biochemistry, Virus Laboratory, University of California, Berkeley.

for about forty minutes; with the barbital buffer a field strength of about 6 volts per centimeter, and a current of 7-8 milliamperes was maintained for sixty-two to ninety minutes. The bath temperature during all of the experiments was 3° C.

The patterns were photographed by the scanning method of Longsworth,[21] and the areas of the peaks were calculated from photographic enlargements by use of a planimeter following the method of Tiselius and Kabat.[22] The total protein was determined by the biuret method as modified by Wolfson and associates.[23] The amount of each protein fraction was calculated from its area in the electrophoretic patterns.

CASE REPORT

M. B., a white woman aged 61 years, who was first seen here in October, 1950, stated that in August, 1949, she first noted swelling of the toes and fingers which later spread to the ankles and legs. This gradually increased in severity. At the onset the patient felt cramping pains in both large toes and rapid beating of the heart with shortness of breath. The diagnosis of rheumatoid arthritis was made, and the patient put on gold therapy. In October, 1950, multiple myeloma was suspected, since Bence-Jones protein was detected in the urine. The patient was then referred to the University of California Hospital where the diagnosis was confirmed by sternal puncture on October 30, 1950, the smear having revealed sheets of plasma cells. The diagnosis was corroborated by electrophoretic analysis of the serum proteins. Biopsy specimens of skin, skeletal muscle and synovial membrane (knee) revealed deposits which stained as amyloid by Congo red. On Nov. 7, 1950, the Congo red test showed a retention of 91 per cent of injected dye in the blood after sixty-five minutes. During the illness the patient complained of joint symptoms. The right shoulder was swollen and restricted in motion. Also, all the fingers were swollen, stiff, and fixed in a flexed position. She was found to have a blood pressure of 170/100 mm. Hg, auricular fibrillation with a pulse deficit (112 apex, 80 radial), dyspnea, and edema. On Jan. 2, 1951, blood study showed hemoglobin 8.5 Gm. per 100 ml., red blood cells 3.2 millions, and white count 7,600 per cubic millimeter. The urine specimens showed Bence-Jones protein on several examinations. Hemoglobin normal numbers of plasma cells persisted in the bone marrow.

Roentgenograms of the skull disclosed no evidence of bone destruction. The thoracic and lumbar spine and the hands showed changes typical of hypertrophic and degenerative joint disease.

Treatment with cortisone (twenty-seven days; 200 mg. per day intramuscularly) gave subjective improvement, manifested by mental alertness and diminished pain and stiffness in the shoulders. Symptoms returned shortly after the cortisone was discontinued. Urethane (first period: eight days, 3.6 Gm. per day; second period: sixteen days, 3.6 Gm. per day) had no influence upon the clinical picture or the laboratory findings. The symptoms gradually increased in severity, and the patient died suddenly on Jan. 12, 1951. *Autopsy diagnosis:* Plasma cell myeloma with amyloid disease involving mesodermal tissue (skeletal and smooth muscle, all layers of the heart, tongue, synovial membranes; walls of vessels in all organs except the central nervous system).

RESULTS AND DISCUSSION

The results are summarized in Table I. In the seventy-three days during which the patient was observed, four electrophoretic analyses were performed, the last shortly before death. Total proteins were low, as shown in Table I. Therefore, this case should be classed as one of the hypoproteinemic type. Abnormal globulins were markedly increased, and migrated within the range of the beta globulins (Fig. 1 and Table I). These continued to rise slightly during the period of observation until death. At the same time the already low albumin level continued to decrease slightly (Table I). The alpha and gamma

globulins showed minimal variation during observation. There was a slight increase in alpha globulins in the sample of Dec. 21, 1950, but this rise did not persist. Therapy with cortisone and with urethane did not induce any notable change in total protein values or in the abnormal peaks in the electrophoretic patterns. The slight increase of the alpha globulins after the first period of treatment with urethane cannot be correlated with this treatment, because the increase did not appear during the second period of treatment.

Comparative study of the patterns obtained with barbital and the citrate-barbital buffer is of interest. Striking differences are seen in the abnormal proteins of the myeloma serum with the two buffers and consists of the following: (1) with the barbital buffer only one abnormal peak is present within the mobility of the beta globulins; (2) the citrate buffer shows two abnormal

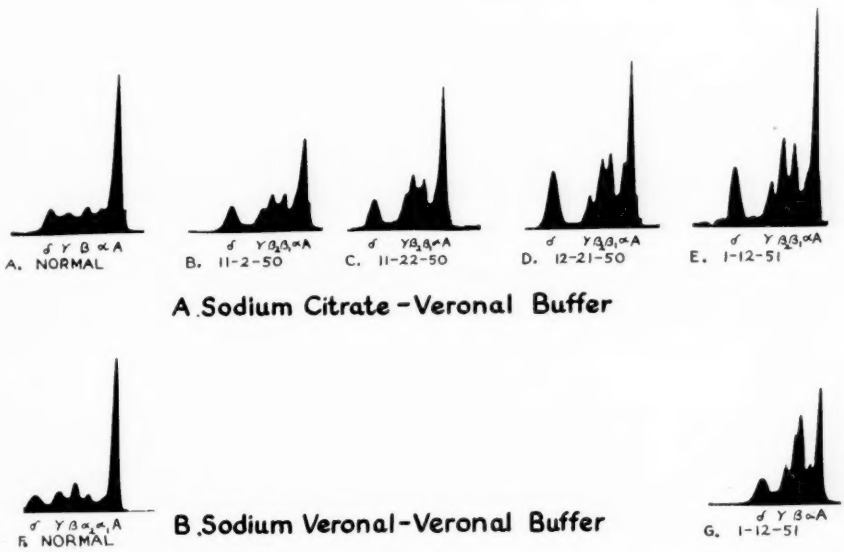

Fig. 1.—Serial electrophoretic patterns in multiple myeloma with amyloidosis. Ascending boundaries.

TABLE I. SERUM PROTEIN* IN MULTIPLE MYELOMA

	DATE OF SAMPLE	ALBUMIN	ALPHA GLOBULIN	BETA GLOBULIN			GAMMA GLOBULIN	TOTAL
				BETA 1	BETA 2	TOTAL		
Sodium citrate—Veronal buffer								
Control		4.08	0.64			0.85	0.85	6.42
Myeloma	11/ 2/50	2.31	0.23	0.78	0.87	1.85	0.48	4.67
Myeloma	11/22/50	2.27	0.26	0.85	0.89	1.74	0.55	4.82
Myeloma	12/21/50	1.47	0.52	0.67	0.75	1.42	0.32	3.73
Myeloma	1/12/50	1.65	0.37	0.82	0.95	1.77	0.44	4.23
Sodium veronal—Veronal buffer								
Control		3.63	0.84			0.75	0.81	6.03
Myeloma	1/12/51	1.55	0.32			1.79	0.57	4.23

*In grams per cent.

peaks of about the same height. These were seen in all four experiments performed with this buffer, and were observed to be increased slightly during the period of observation.

No conclusion can be drawn regarding the relation of the two abnormal electrophoretic peaks to the deposition of amyloid in this case. It would be interesting to make a comparative analysis similar to that carried out by Gutman with Bence-Jones protein.[4] This might give some indication of the nature of the amyloid substance and show its relationship to Bence-Jones protein.

SUMMARY

1. A case of beta-myeloma with hypoproteinemia has been studied.

2. Repeated electorphoretic studies of the serum showed a progressive rise in the proportion of abnormal protein with a slight decrease in the albumins accompanying it.

3. Cortisone and urethane had no influence upon the clinical picture or the abnormal electrophoretic pattern.

4. By using the sodium citrate-veronal buffer the electrophoretic pattern showed two abnormal beta-globulin peaks, whereas only one beta-globulin peak could be detected in an experiment with the veronal buffer on the same sample.

5. In view of these two peaks it is proposed to classify this case as a "beta-one beta-two myeloma."

6. No conclusion is drawn concerning the relationship of the abnormal beta one and beta two globulins to the deposition of amyloid in this case.

The authors express their appreciation to Miss Patricia Jenks and Mr. Robert Brooks for their technical assistance.

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INVESTIGATIONS ON THE RELATIONSHIP BETWEEN THE AGE OF INDIVIDUALS AND THE AGGLUTINABILITY OF THEIR RED BLOOD CELLS

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FEW investigations have so far been reported concerning the effect of the age of a subject upon the agglutinability of his red cells.

Kemp (1930) investigated this relationship with respect to the agglutinogens A and B, and found that the agglutinability of the red cells was first detected in a fetus 37 days old, and then slowly increased to the age of 20 years, remaining unchanged thereafter.

Schiff and Hubener (1926) investigated the agglutinability of the red cells with respect to A and B agglutinogens, and demonstrated varying individual sensitivity, not primarily related to the age of the donor.

The above authors estimated agglutinability by titrating the cells with the appropriate serum, and noting the final titer at which agglutination could still be seen.

The present investigation is primarily aimed at estimating the agglutinability of Rh positive red cells by the Rh agglutinin anti-D in subjects from the age of 17 years and older. Donors were divided into ten age groups from 16 to 65 years.

Cells from 100 individuals were phenotyped at the start of the investigation in order to be able to assess the effect (if any), of a "double dose" of the antigen D upon the agglutinability of red cells by anti-D. Cells of phenotypes R_1R_1, R_1R_2, and R_1r (as defined by anti-D, C, E and e) were used. Thirty-nine cells belonged to the R_1R_1 or R_1R_2 phenotype, i.e., double D dosage, and forty-nine cells belonged to the R_1r phenotype, i.e., single D dosage. The remaining cells belonged to the phenotype R_2R_2 or R_2r, and thus had to be excluded as no anti-e serum was available.

The mean agglutination score of the double dose cells was 36.6, and that of the single dose 36.1. This difference is not significant.

The agglutinability of the red cells was estimated by titrating them with the same anti-D serum throughout, giving each degree of agglutination an appropriate score and taking the sum of these scores as a measure of the agglutinability of the cell.

This method takes into consideration not only the titer at which agglutination ceased, but also the intensity with which the cells agglutinated at different titers.

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MATERIALS

1. Saline anti-D serum (laboratory serial number "482 absorbed") with a titer of 1:32. This was derived from a donor of group B and the anti-A antibody had been absorbed before use. (The serum was kept at −20° C. when not in use.)

2. Anti-D, E, C, and c serum (two of each).

3 Control cells from author (L.K.M.) phenotype R_2r.

4. Blood from donors of ages from 17 to 65 years.

The blood of subjects from 20 to 65 years old was derived from blood donors attending Sessions in the Sheffield Region and taken at random for testing. The blood was collected from a vein into a dry tube and kept at 4° C. until tested fourteen to twenty hours afterward.

The cells from donors of ages below 20 years were mainly obtained from the laboratory staff by pricking the ear and collecting about 1 ml. of blood into a dry tube which was kept at 40° C. until used fourteen to twenty hours afterward.

METHOD

About 15 to 25 samples were tested at one sitting.

The following steps were taken in the investigation:

1. An accurate 2 per cent cell suspension in saline was made from the blood of L.K.M., fresh blood being used each time.

2. An approximate 2 per cent cell suspension was made from the cells under investigation, by diluting the blood with saline until the color of the test suspension was equal to that of the control suspension.

3. Titrations were made with anti-D serum (482 absorbed) against the control cells and the cells under investigation by the precipitin tube technique, as follows:

Dilutions from 1:1 to 1:512 were made from the serum in ten master tubes.

One drop of each dilution was placed in precipitin tubes, the number depending on the number of titrations being made at the time.

One drop of the cell suspension was then placed in each of the ten tubes containing the different dilutions of the anti-serum.

The tubes were placed in the incubator at 37° C. for one and three-fourth hours.

The cells from each tube were removed with a Pasteur pipette, transferred to a glass slide and the amount of agglutination that had taken place observed microscopically.

When cells from donors of group A were tested, an absorption control, i.e., AB Rh negative cells, was put up against the serum.

The following agglutination symbols and their corresponding scores were employed.

V	= Large visible agglutinates	= 9
(V)	= Small visible agglutinates	= 8
+	= Large microscopical agglutinates	= 7
(+)	= 12-20 cells agglutinated	= 5
GW	= 7-12 cells agglutinated	= 3
W	= 2-7 cells agglutinated	= 1

The control cells were always titrated and read first. Their agglutination scores in 12 titrations were found to range from 40 to 48, i.e., 48, 42, 44, 45, 42, 40, 42, 40, 45, 44, 45, 44.

A score of 45 was taken as the arbitrary mean score of the control cells. When the score of the control cells differed from this number, the appropriate alteration was made in the score of the test cells titrated at the same time, e.g., when control score was 40, the score of the test cells was multiplied by 45/40.

Altogether 203 cells were titrated.

The frequency distribution of the agglutination scores in the ten age groups is shown in Table I.

TABLE I. FREQUENCY DISTRIBUTION OF THE AGGLUTINATION SCORES IN TEN AGE GROUPS

SCORES / AGE GROUPS	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	NO. TESTED	MEAN SCORES OBSERVED	MEAN SCORES CALCULATED
16-20					1						2		1	1		1		1		1		1		1		1		10	39.6	40.0
21-25								1				2		1	4	1	1	4	2			1					1	18	40.6	39.2
26-30				1			1	1	1		2	2	1	2	5	2	1	1	2									22	37.4	38.4
31-35	1					2				2	4	4	2	2	3	1	1	1	1									24	36.3	37.6
36-40			1	1			1		1	1	3		3	5	3	1	1	1	1	1	1		1					26	37.6	36.8
41-45								4	2	1		4	1	1		1	1	2										17	36.0	36.2
46-50					1	1	3	1		1	2	5	1		1	1			1			1	1					20	36.0	35.2
51-55			1	2	2	2	5		1	3	2	4		2	1	1	2					2						30	34.4	34.6
56-60	1					5	3	2	3	6	1	1	1						1				1					25	33.0	33.6
61-65					1	2	3	2		1	1		1															11	32.0	33.0
Totals	2	-	2	4	5	12	16	11	8	15	17	22	11	14	17	9	7	10	8	2	3	3	3	-	1	-	1	203		

Estimated regression line of agglutination score on age. Score = 36.18 - 0.162 (age: 41.45)
Estimated regression coefficient of score on age. b = 1.62 ± 0.236 units per 10 years.
Estimated standard deviation about the regression line. = 4.39 units.

The regression line for score on age has been fitted by the method of least squares. There is shown a decrease in the agglutination scores of Rh positive red cells tested with the same anti-D serum from the age of 20 to 65 years, with a decrease of 1.623 ± 0.236 in average score per ten years. The decrease can obviously not be dué to chance alone. If the variance is analyzed within and between age groups it can be shown that the variation with age is adequately accounted for in these data by the straight regression line, i.e., a significantly better fit would not be obtained by fitting any other curve to the observations. Mean age—specific scores as calculated from the regression formula have been placed alongside the observed means.

CONCLUSIONS

An investigation to determine the agglutinability of Rh positive red cells by anti-D serum in relation to the age of the subject is described.

The results indicate a general downward trend in agglutinability from 20 to 65 years of age associated with individual variation in the sensitivity of cells, not related to age.

Certain practical considerations emerge from this investigation:

1. The titer of an anti-D serum is influenced not only by the age of the subject whose cells are used in the titration but also by other individual differences in agglutinability.

2. In controlling a known anti-D serum, where weakly-reacting D positive cells are required, suitable cells are most likely to be found in older persons of the appropriate phenotype.

3. In testing an unknown serum for the presence of anti-D or in excluding the presence of anti-D in a serum possessing anti-C suitable strongly-reacting cells are most likely to be found in young persons of the appropriate phenotype.

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STUDIES ON PLASMA LIPOPROTEINS IN NERVOUS SYSTEM DISEASES

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GOFMAN and his associates[1, 2] recently introduced new methods for classifying and studying plasma lipoproteins with the aid of the ultracentrifuge. These investigators were particularly interested in the relationship of cholesterol and specific lipoproteins to the etiology of atherosclerosis. Since nervous tissue contains appreciable amounts of uncharacterized insoluble lipid complexes, it was felt that patients with diseases of the central nervous system might afford an excellent opportunity for investigating abnormalities in the distribution of plasma lipoproteins. In the present investigation the chemical and physical characteristics of plasma lipoproteins of patients with neurologic disturbances and psychoses were studied by utilizing flotation procedures in the ultracentrifuge.

METHODS

Male medical students and staff members (18) served as controls. Patients were divided into the following groups: (a) neurologic cases with and without complications in an age range of 12 to 74 years, consisting of 44 males and 24 females; and (b) psychotic patients with and without complications in an age range of 21 to 77 years consisting of 12 males and 6 females. The diagnoses are not listed, since they are not pertinent.

Approximately 50 ml. of blood were drawn before breakfast into a heparin-moistened (Liquaemin, Organon, Inc.) syringe. The sample was immediately centrifuged, and the plasma removed. About 15 to 25 ml. of plasma was prepared for flotation in the ultracentrifuge.

The procedures recommended by Gofman and co-workers[1] for the flotation of lipoproteins were followed. Plasma was diluted with an equal volume of 17.6 per cent NaCl solution to yield a mixture with a density of about 1.0625. This mixture was centrifuged at 56,100 r.p.m. for three and one-half hours at room temperatures (20° to 30° C.) in the preparative rotor of the Spinco (Model E) ultracentrifuge. The top lipid-rich layer was removed with a syringe and a hypodermic needle which was hooked at the end. The lipoproteins of the top fraction were analyzed in the analytical rotor of the Spinco ultracentrifuge and classified according to their flotation rates, as recommended by Gofman and associates.[1] All analyses were completed within twenty-four to thirty-six hours after the blood was drawn. Analytical data for the top fractions are expressed in terms of their concentrations in 100 ml. of original plasma.

Experiments were conducted with the Spinco Model E and Model L ultracentrifuges to determine the optimum conditions for flotating the lipoproteins of plasma. Electrophoretic analysis of the various layers was used as a measure of the degree of separation. According to the results it appeared that three and one-half hours' centrifugation at 56,100 r.p.m. was sufficient to yield excellent flotation of the lipoproteins. On visual inspection the lusteroid tube contains a top lipid layer from 0.3 to 0.6 cm. in depth over a clear layer about 0.3 to 0.5 cm. in depth. The intensity of the color of the remaining residual solution changes

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from straw at the top to yellow at the bottom of the tube. It was impossible to obtain good electrophoretic patterns of the top lipoprotein layer. However, in the majority of cases no resolvable sedimenting component was present in the ultracentrifuge pattern of the top solution. In some cases a maximum of 1 per cent of the total pattern area could be attributed to a protein which appeared to be albumin. By electrophoretic analysis the under-

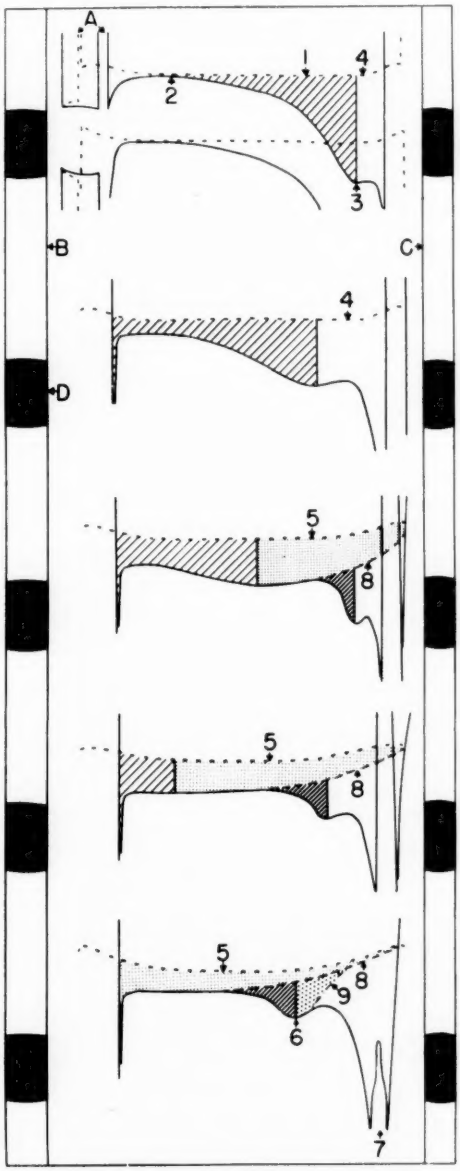

Fig. 1.—Ultracentrifuge patterns of a top solution selected to illustrate the procedure for analyzing component areas. Top four tracings after 0, 6, 15, and 27 minutes at rotor speed of 39,470 r.p.m. Bottom pattern immediately after rotor attained 50,740 r.p.m. *C* and *D* refer to outside reference line and the dummy cell opening, respectively. Remaining designations explained in text. (Fisk, Berry, and Chanutin.)

lying clear layer appeared to be free of protein. The top portion of the residual solution contained minimal amounts of a_1-globulin, and albumin. At successive lower depths of the residual solution, increasing amounts of all the globulins and albumin could be determined.

The analytical rotor was first brought to a speed of 39,460 r.p.m., and the pattern was examined for the presence of a rapidly flotating component. If this component was present, pictures were taken at one- or two-minute intervals until the peak disappeared. The speed of the rotor was increased to 50,740 r.p.m., and photographs were taken at intervals of four, eight, or sixteen minutes, depending on the complexity of the observed pattern. A series of photographs of patterns for 8.8 per cent saline, obtained at 39,460 and 50,740 r.p.m., served as controls for the force-field as related to time. The opening of the light source slit and the position of the angle bar were kept constant during the entire experimental period.

Analysis of Patterns.—It is essential to utilize the correct saline pattern to serve as the base line for the precise analysis of the ultracentrifuge patterns. The optical system of the Spinco centrifuge causes the pattern to appear as a broad band. With the aid of an enlarger, the upper lines of the lipoprotein and saline patterns are traced for determining flotation rates and areas of the different components.

It is necessary to modify the conventional planimeter method of measurement in order to obviate the effect of the boundary spreading of each component. The procedures followed are illustrated in Fig. 1. The initial boundary of the S_f 30-70 component *1* begins to spread immediately. The beginning of its boundary *2* approaches the meniscus line *A*. Usually the main portion of the component is at the center of the cell at this time. The first component *1* is partitioned by drawing a line parallel to the inside reference line *B* from the peak *3* to the saline base line *4*. The area of the initial peak in front of the dividing line is an accurate "half boundary" since it is not influenced by succeeding components. The total area of the initial boundary is obtained by doubling the value of the "half boundary." Despite the disappearance of the S_f 30-70 peak at 50,740 r.p.m., the spreading of the boundary *5* may be pronounced and therefore affects the succeeding S_f 0-30 areas *(6, 7)*. In order to determine the effect of this boundary spreading on the succeeding areas (S_f 20-30, S_f 10-20, or S_f 0-10) a curve *8* is drawn from the maximum elevation of the boundary of the initial component to the saline base line so that the planimeter value is the same as that of the "half boundary" area. This curve represents the corrected base line for the next flotating component. A line is drawn parallel to the inside reference line from the peak of the second boundary to the corrected base line *6* and the "half boundary" is measured. The trailing side of this second boundary *9* is drawn to provide the corrected base line for the succeeding boundary. These procedures are followed for each component. Agreement of the values for areas in successive photographs of patterns of a given sample proves the reliability of the procedures used.

The effect of boundary spreading on the areas of the flotating components is of considerable importance. In order to obtain accurate results, it is essential to have a number of patterns available for analysis.

Chemical Procedures.—The total lipides of the plasma and the ultracentrifuge fractions were extracted with hot acetone-absolute ethanol (1:1). Aliquots were analyzed for total and free cholesterol according to the procedures of Sperry and Brand.[3] The acetone-ethanol extract was evaporated to dryness, immediately extracted with petroleum ether, filtered and brought to volume. Aliquots were analyzed for phospholipid.[4]

RESULTS

Chargaff and co-workers[5] found that the β-globulin anomaly of the electrophoretic pattern of plasma disappeared in the presence of large amounts of heparin (400 mg. per cent). These workers assumed that the heparin reacted with the labile β-globulin fraction and thus liberated bound lipides. During the course of studies with plasma, it was observed that the electrophoretic pattern

was not influenced by small amounts of heparin (1.4 mg. per cent) but at higher concentrations (20 mg. per cent) the α_2-β region was affected. Experiments were conducted with aliquots of oxalated plasma adjusted to 0, 1.4 and 20 mg. per cent heparin, and it was found that neither the distributions nor the concentrations of the various S_f components of the top flotated ultracentrifuged fraction were affected.

The frequency distribution of flotation rates for every component that could be resolved between S_f 0 and S_f 70 in the ultracentrifuge patterns of the top fraction for all cases is shown in Fig. 2. According to this distribution, it appears feasible to divide the components into the S_f classes 0-10, 10-20, 20-30, and 30-70 as suggested by Gofman and associates.[1] The curve between S_f 10 and S_f 20 is asymmetrical and could therefore be subdivided into S_f 10-15 and S_f 15-20 c'asses.

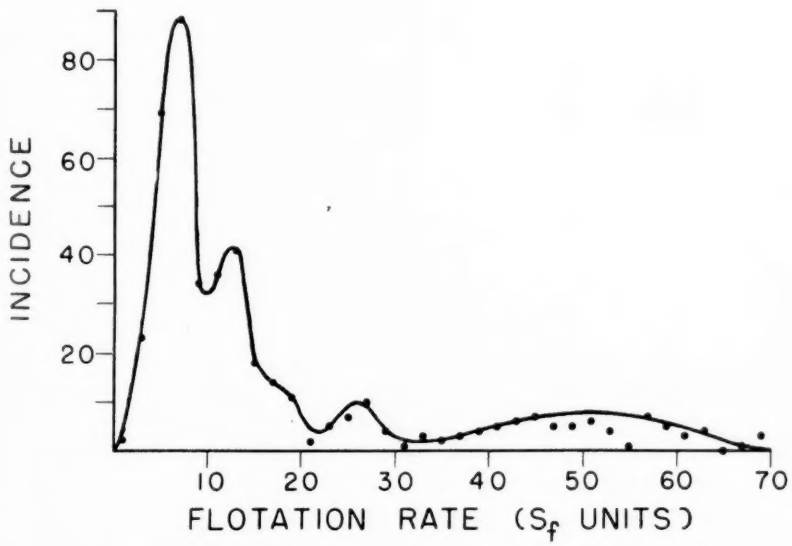

Fig. 2.—Frequency distribution of flotation rates of lipoprotein components. (Fisk, Berry, and Chanutin.)

The percentages of the control, neurologic, and psychotic groups whose plasmas contained varying concentrations (absolute planimeter units) of characterized lipoproteins are shown in Fig. 3. It can be seen that the distribution of the lipoprotein components are essentially the same in the three groups studied. Values of Chi-square* which test the independence of the neurologic and psychotic groups from the control group do not show significant differences. All plasmas contain the S_f 0-10 groups; the S_f 10-20, S_f 20-30, and S_f 30-70 groups are present in about 70, 20, and 50 per cent, respectively, of the samples analyzed.

*Chi-square values were calculated for a fourfold table which contained the number of control individuals above and below the control means and the number of individuals in the respective patient groups above and below the control mean. The 95 per cent probability limit was set for the Chi-square value.[6]

The cholesterol and phospholipid concentrations of the plasma and the top fraction in the three groups of subjects are graphed in Fig. 4. No group differences are noted for these lipide constituents by Chi-square analysis. The elevated values for cholesterol and phospholipid in the plasmas and the top fractions observed in a number of cases do not appear to be typical for any specific disorder.

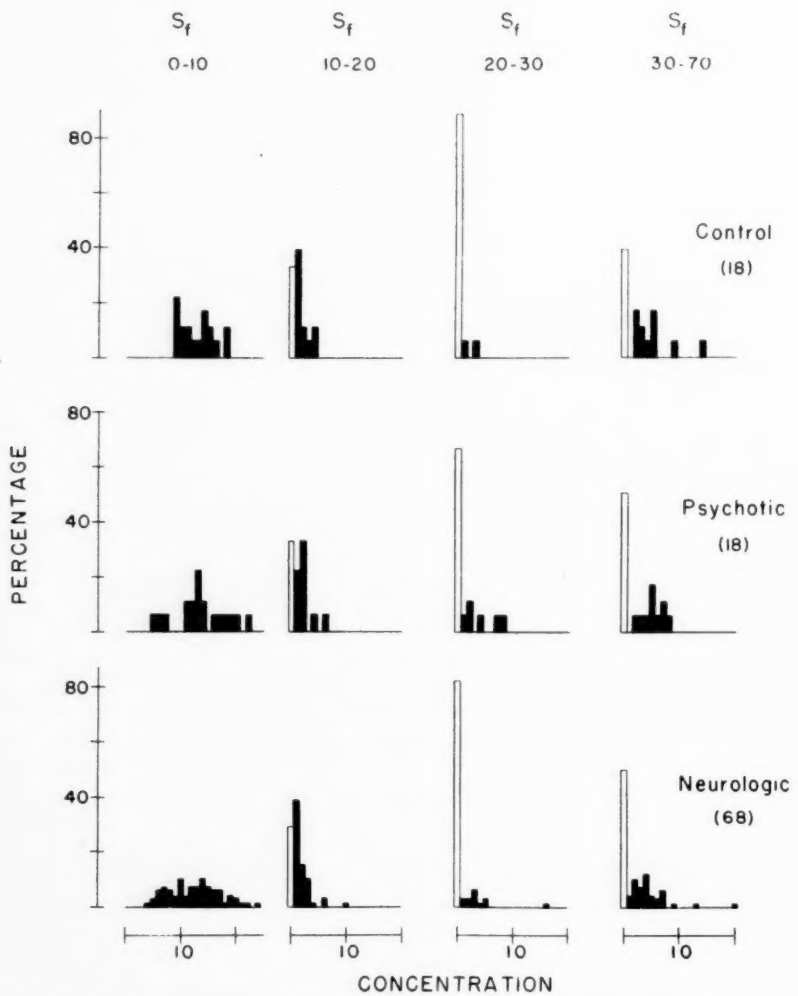

Fig. 3.—The distribution of the various lipoprotein groups in control neurologic and psychotic patients.

The unshaded columns represent the percentage of cases in which the respective components are absent. (Fisk, Berry, and Chanutin.)

The top fraction contains 36 ± 3.3 per cent and 52 ± 3.7 per cent of the total plasma phospholipid and cholesterol, respectively, in 119 cases. According to statistical analysis these values are representative for each of the 3 groups.

The mean values for the percentage of free cholesterol in the plasmas and in the top fraction of the respective groups vary within the control range of 26 to 30 per cent.

The partial correlation coefficients[7] or the top fraction phospholipid or cholesterol and the absolute planimeter values of the areas of the various S_f classes of the top fraction are presented in Table I. These analyses represent the correlation between paired data in all cases.

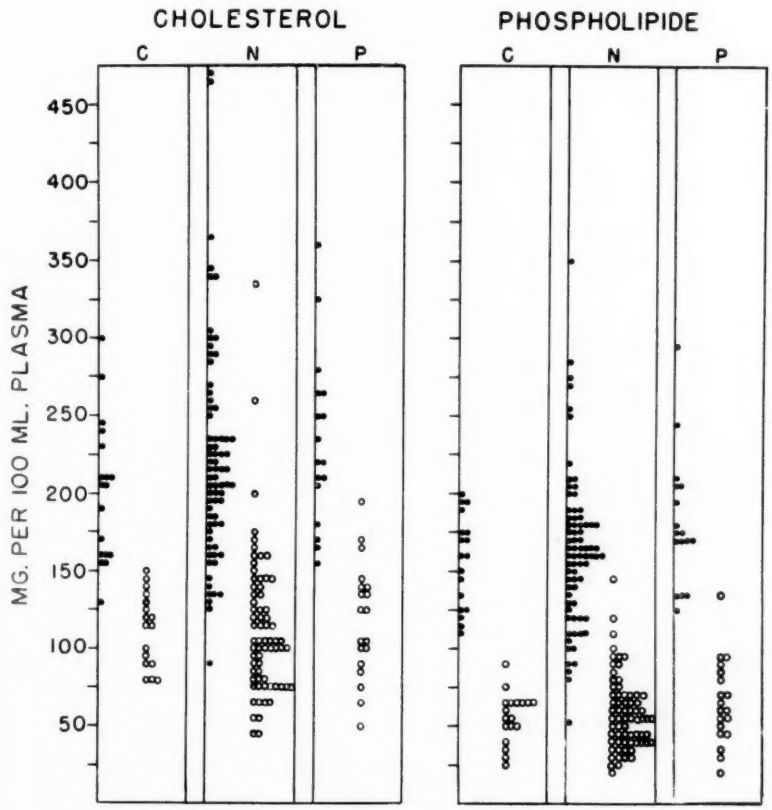

Fig. 4.—Scatter diagrams for cholesterol and phospholipide concentrations in the control (C), neurologic (*N*) and psychotic (*P*) groups. Values for plasma (solid circles) and top ultracentrifuged fraction (open circles) are presented. (Fisk, Berry, and Chanutin.)

TABLE I. PARTIAL CORRELATION COEFFICIENTS FOR THE CONCENTRATIONS OF LIPIDES AND ULTRACENTRIFUGAL COMPONENTS OF THE TOP FRACTION

PHOSPHOLIPID (102)			CHOLESTEROL (103)		
S_f CLASS	R	95% LIMIT	S_f CLASS	R	95% LIMIT
0-10	0.92	0.88-0.94	0-10	0.71	0.58-0.80
10-20	0.39	0.21-0.56	10-20	0.25	0.05-0.44
20-30	0.30	0.10-0.47	20-30	0.18	0.03-0.37
30-70	0.61	0.45-0.71	30-70	0.58	0.43-0.71

Figures in parentheses represent number of paired analyses.

The correlation coefficient of 0.92 for phospholipid and area (concentration) of the S_f 0-10 class is high. This analysis indicates that the phospholipid concentration per unit mass of the S_f 0-10 class is greater than found in the remaining S_f groups. A relatively high correlation coefficient of 0.61 is obtained for the S_f 30-70 class. The coefficients for the S_f 10-20 (0.39) and the S_f 20-30 (0.30) groups are comparatively low.

The correlation coefficient for the cholesterol and the S_f 0-10 area is 0.71. The values of 0.25, 0.18 and 0.58 are obtained for the S_f 10-20, S_f 20-30 and S_f 30-70 classes, respectively.

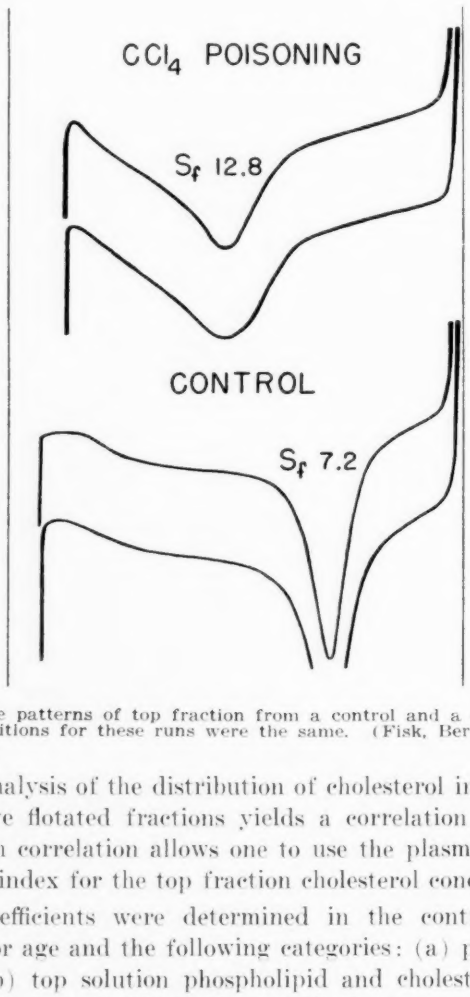

Fig. 5.—Ultracentrifuge patterns of top fraction from a control and a case of CCl_4 poisoning. Experimental conditions for these runs were the same. (Fisk, Berry, and Chanutin.)

A statistical analysis of the distribution of cholesterol in samples of plasma and their respective flotated fractions yields a correlation coefficient of 0.74. This relatively high correlation allows one to use the plasma cholesterol values as a fairly reliable index for the top fraction cholesterol concentration.

Correlation coefficients were determined in the control neurologic and psychotic groups for age and the following categories: (a) plasma phospholipid and cholesterol; (b) top solution phospholipid and cholesterol; and (c) concentrations of the S_f 0-10, 10-20, 20-30, and 30-70 components. The statistical results indicated almost complete lack of correlation of these various categories with age. It may be concluded that age is not a significant factor influencing the results obtained in these experiments. Insufficient data were available for

assessing the influence of sex. Russ, Eder, and Barr[8] observed that there were comparatively small differences in the concentrations of the lipid components in the plasmas of normal males and females.

Analyses of plasmas of patients with medical or surgical disorders yielded results which were essentially the same as those reported for the neurologic and psychotic groups. The ultracentrifuge pattern of the top fraction (Fig. 5) of a patient, severely intoxicated with CCl_4, is shown because the lipoprotein components of the S_f 0-10 group are not demonstrable. Analyses of this plasma yielded the following values: total cholesterol 151 mg. per cent; per cent free cholesterol, 42 per cent; phospholipid 185 mg. per cent.

DISCUSSION

In Fig. 1 it is seen that in the presence of the S_f 30-70 group, the original saline base line cannot be utilized in the measurement of the S_f 20-30 and S_f 10-20 groups. If the original base line is used, erroneously high concentrations will be obtained. Since a good correlation (0.58) exists between cholesterol concentrations and S_f 30-70 area, it follows that the correlation coefficients for the two succeeding groups would be increased, if the influence of the S_f 30-70 group is not eliminated.

A great deal of interest has developed in the work of Gofman and his colleagues who postulate that lipoproteins of the S_f 10-20 class are associated with the development of atherosclerosis. These workers state that these molecules are rich in cholesterol.

Key's[9, 10] analysis of the uncorrected data of Gofman yielded a correlation coefficient of the order of 0.6 between the plasma cholesterol and the S_f 10-20 class. In the present experiment the partial correlation coefficient for the top fraction and the S_f 10-20 class of 0.25 is much lower (Table I). A comparison with Key's analysis is permissible since a good correlation (0.74) between the cholesterol concentrations of the top fraction and of the plasma was found in the present studies. Since the cholesterol concentrations of the S_f 10-20 group is low, these molecules are probably not directly responsible for the deposition of cholesterol in such conditions as atherosclerosis.

This investigation does not reveal any striking or consistent changes in the lipid metabolism of patients with neurologic or psychotic abnormalities. It must be emphasized that precise methods are not available for characterizing the large number of plasma lipoproteins which have different mass, density and shape. As a matter of convenience, it is desirable to characterize the lipoprotein molecules in terms of relatively large families, but under these circumstances it is difficult, for example, to assess the significance of individual components having flotation rates varying between S_f 1.9 and 10. This problem is further complicated by the fact that practically nothing is known concerning the function and origin of the lipoproteins of plasma and tissues.

SUMMARY

The plasma lipoproteins of healthy young males and of patients with neurological diseases and psychoses were fractionated by flotation in the ultracentrifuge and analyzed by chemical and physical procedures.

Methods for analyzing the areas of the lipoprotein groups in ultracentrifuge patterns are described.

Data are presented for the distribution of phospholipid and cholesterol in plasma and in the flotated top fraction.

Partial correlation coefficients indicate that the S_f 0-10 and S_f 30-70 lipoprotein groups contain the greatest amounts of total cholesterol and phospholipid. The correlation coefficients for the S_f 10-20 and S_f 20-30 groups are of a low order. The significance of these findings is discussed.

The distribution and concentration of plasma lipoproteins are not affected in patients with neurologic or psychotic disorders.

The authors are grateful to Mr. Lionel Weiss for his advice and assistance in computing the statistical data, to Mr. T. J. Boyd for his technical assistance, and to Dr. W. O. Klingman of the Department of Neurology and Psychiatry for his cooperation in procuring the bloods for this work.

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HISTOCHEMICAL DEMONSTRATION OF INTRAVENOUSLY INJECTED DEXTRAN IN KIDNEY AND LIVER OF THE MOUSE

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PARTLY hydrolyzed dextran, a water-soluble polysaccharide composed of glucose units, has been advocated as a plasma substitute for the treatment of shock.[1-3] Wider acceptance of dextran* for this purpose is limited by the uncertainties of its distribution in the tissues and its ultimate fate. Some investigators have assumed from the results of chemical assays that no storage of dextran occurs and that it is probably metabolized as glucose.[1-2] But, Bull and his co-workers obtained serologic evidence of dextran storage in lymph nodes and spleen of rabbits up to fifty-six days after infusion.[3] If dextran is degraded and eventually metabolized, the organ sites and enzymatic means of these processes are unknown. Some workers have observed morphologic changes in tissues of experimental animals given intravenous dextran[4-6]; others have not.[1-3] Staining of dextran in tissue sections has apparently been unsuccessful.[4-5] A method for the demonstration of dextran in tissue sections should permit study of its fate in the body at the cellular level. Furthermore, such a method might clarify the pathogenesis of morphologic changes believed due to dextran, as well as disclose changes hitherto unrecognized.

Tissues of mice given dextran intravenously after experimental burn shock have shown two morphologic changes not present in either untreated or saline-treated control mice.[6] Stained sections of kidneys have shown dilation of tubules and flattening of epithelium in dextran-treated mice. Liver sections have shown vacuolization of parenchymal cells due neither to glycogen nor to fat. It seemed possible that these changes could be the result of local accumulations of dextran. No dextran was demonstrable by a variety of stains. In the course of ordinary histologic processing there are many opportunities for contact of tissues with water that could cause loss of dextran by solution. Attention was directed, therefore, to measures that might prevent such losses.

MATERIAL AND METHODS

Dilute aqueous solutions of dextran (Commercial Solvents Corp.) were streaked on glass microscope slides and allowed to evaporate. Multiple tests showed the residue to be insoluble in 70 to 95 per cent alcohol. The briefest exposure to water, however, resulted in complete solution of such films and spots of evaporated dextran. Next, dextran-streaked slides were subjected to the periodic acid-Schiff histochemical reaction, independently introduced by

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*Dextran is used in this paper to refer to the partly hydrolyzed or clinical product.

McManus,[7] Lillie,[8] and Hotchkiss.[9] To avoid the total solution of dextran residue that occurred with the aqueous periodic-Schiff reagents, it was necessary to modify the reagents by use of 70 per cent alcohol as the solvent in place of water. When alcoholic reagents were used, the periodic-Schiff reaction of dextran was intensely positive. Dextran residue was colored deep purple. These results suggested that if prior exposure to water were avoided dextran might be stained in tissue sections by the alcoholic periodic-Schiff method.

The schedule of tissue preparation and section cutting adopted after some trials was as follows: (1) fix thin blocks of tissue in absolute alcohol at –5° C. for about forty-eight hours; (2) transfer to a fresh change of absolute alcohol for eighteen to twenty-four hours; (3) clear in paraffin solvent, infiltrate, and embed in paraffin; and (4) cut at 5 microns without exposure of block or sections to water. Float sections on warmed 95 per cent alcohol and mount on glass slides. Drain slides quickly and dry in paraffin oven before staining.

The periodic-Schiff staining routine for water soluble, alcohol insoluble substances was as follows: (1) take sections through xylol and alcohols to 70 per cent alcohol; (2) treat sections for ten minutes in 1 per cent periodic acid (H_5IO_6) dissolved in 70 per cent alcohol; (3) rinse in 70 per cent alcohol, for five minutes; (4) place sections in alcoholic Schiff reagent for fifteen minutes. Reagent is prepared by combining 11.5 ml. of aqueous leucofuchsin (Schiff reagent), 0.5 ml. concentrated HCl, and 28 ml. of absolute alcohol. Lillie's Schiff reagent (one gram per cent) is recommended because of its high fuchsin content[10]; (5) rinse in three five-minute changes of alcoholic sulfite solution, freshly prepared by adding 0.5 Gm. sodium metabisulfite ($Na_2S_2O_5$) and 1 ml. concentrated HCl to 99 ml. 70 per cent alcohol; and (6) rinse in 70 per cent alcohol for five minutes. Counterstain with alcoholic dye solutions if desired, dehydrate, clear, and mount. The alcoholic solution of periodic acid remains suitable for use as long as it is colorless. Deterioration is indicated by the appearance of a brown color, presumably due to iodine. The alcoholic Schiff solution preferably is prepared just before use. It soon becomes pink, but retains its staining specificity for several days.

Female mice belonging to the white Swiss, N.I.H. strain and weighing 18 to 22 Gm. were employed for all experiments. Experimental burn shock was produced by immersion of mice in water at 70° C. for 5.5 seconds, using the technique previously reported.[11] Dextran was injected as a 6 per cent solution in 0.9 per cent NaCl into the tail vein. Animals given dextran were sacrificed five hours later, an arbitrarily chosen time interval for determining consistency of results. All mice were fasted overnight previous to the experiment. This was for the purpose of reducing glycogen stores in the liver, thereby facilitating the recognition of dextran in sections.

EXPERIMENTAL

(A) About one hour after induction of experimental burn shock, eleven mice were each injected with 1 ml. of dextran. An equal number of mice received 1 ml. each of 0.9 per cent NaCl. Six hours after the burn, all mice were sacrificed. Tissue blocks of kidney and liver from each animal were processed and stained as described.

(B) Ten normal mice were each given 1 ml. of dextran. An hour later, five of these received a second 1 ml. each. Both groups were sacrificed along with eight untreated normal mice. Liver and kidney were examined from each.

(C) Because of differences observed in the results of experiments *A* and *B*, tissues were taken from five mice given 1 ml. each of dextran after *burn shock* and from five *normal* mice given the same dose of dextran.

RESULTS

(A) After alcoholic periodic-Schiff staining, sections of both kidney and liver from each dextran-treated mouse showed distinctive accumulations of

intensely purple material. These accumulations were not present in simultaneously stained sections of saline-treated mice. Moreover, the purple material was absent in duplicate sections of dextran-treated mice when stained by the aqueous periodic-Schiff routine. Any exposure to water prior to alcoholic periodic-Schiff staining prevented coloration of the material, demonstrating its free solubility in water.

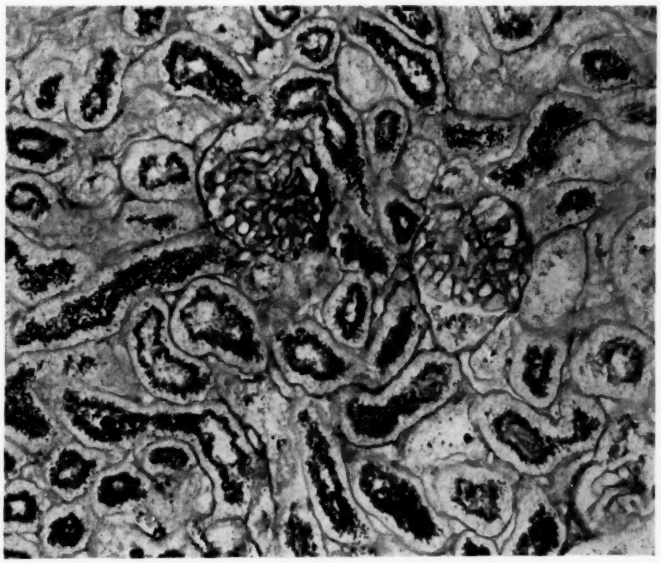

Fig. 1.—Cortex of kidney from mouse given dextran after burn shock, alcoholic periodic-Schiff stain. Granules of dextran are present in the cytoplasm of proximal convoluted tubules. Some occurs in the lumina. (×400.)

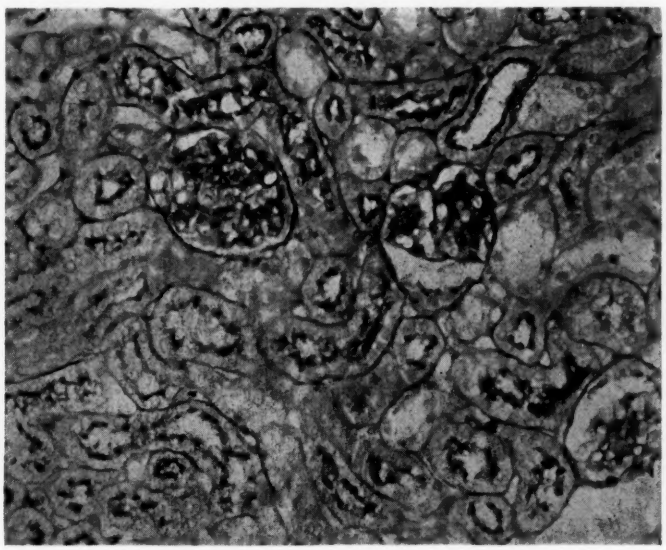

Fig. 2.—Consecutive serial section from tissue block shown in Fig. 1, aqueous periodic-Schiff stain. Note loss of dextran. (×400.)

Deposits of the purple staining material observed in dextran-treated mice were more striking and abundant in the kidney, where they occurred in blood vessels, in tubular epithelial cells, and in excretory passages. The lumina of blood vessels including glomerular tufts contained varying amounts of fine to coarse globules of dense purple material. In all dextran-treated mice, the cells of the proximal convoluted tubules contained numerous purple granules, typically situated in the cytoplasm just beneath the brush borders (Figs. 1 and 2). Casts composed of the dense purple material were observed at all levels of the nephron, but were most numerous in the collecting and distal convoluted tubules (Figs. 3 and 4). In the collecting and distal tubules the casts were generally homogeneous, deeply colored, and filled the lumina. In

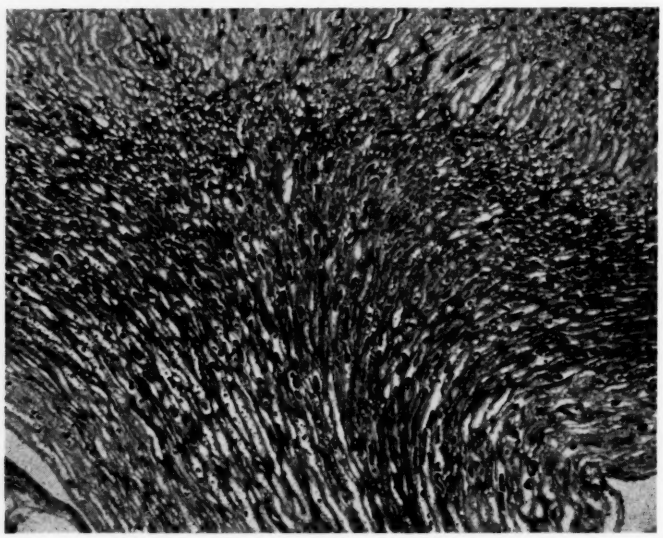

Fig. 3.—Medulla of kidney from same mouse, alcoholic periodic-Schiff stain. Dextran casts in collecting tubules appear black in photograph. (×78.)

more proximal portions of the nephrons, many dense accumulations of the purple material were present, but these were not so often formed or homogeneous. There was frequently a coating of dense purple globules over the mucosa of the kidney pelvis. In alcoholic periodic-Schiff stained sections of saline-treated mice, only the brush borders and basement membranes were colored—exactly as in sections stained by the aqueous periodic-Schiff routine.

Alcoholic periodic-Schiff stained sections of liver from dextran-treated mice contained fairly numerous homogeneous masses of dense purple material within liver cells. Blood vessels contained fine to coarse globules of similar purple material. The cytoplasmic deposits of purple material in dextran-treated mice corresponded to intracellular vacuoles evident in duplicate sections after aqueous staining. Neither such deposits nor such vacuolization were present in sections of liver from saline-treated mice.

(B) Examination of alcoholic periodic-Schiff stained sections of *kidneys* from normal mice given dextran showed some water soluble periodic-Schiff positive granules in most vascular spaces, but only rare casts and no definite cytoplasmic granules. Similarly stained sections of *liver* contained intracellular deposits of purple material that were generally similar but sometimes smaller than those seen after burn shock.

Although sections from normal mice given 2 ml. of dextran showed consistently greater amounts of the water soluble periodic-Schiff positive material, there were only rare casts and no cytoplasmic granules in renal tubules. Slight amounts of finely granular purple material were present in many collecting and distal convoluted tubules. Study of alcoholic periodic-Schiff stained sections of mice not given dextran did not show water soluble periodic-Schiff positive material in blood vessels, renal tubules, or liver cell cytoplasm.

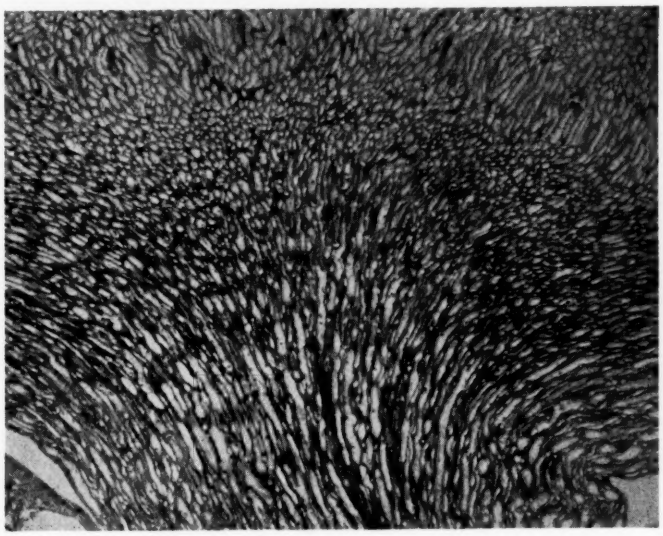

Fig. 4.—Consecutive serial section of tissue block shown in Fig. 3, aqueous periodic-Schiff stain. Dextran casts are absent. (×78.)

(C) Whether mice had been in shock or not at the time of dextran infusion made no consistent difference in the amounts of water soluble periodic-Schiff positive material demonstrable in sections of liver. However, sections of kidney from each mouse given dextran after burn shock revealed extensive deposits, identical to those obtained in experiment *A*. With one exception, kidney sections of normal mice given dextran resembled those observed in experiment *B*. The kidneys of one normal mouse given 1 ml. dextran showed amounts of water soluble periodic-Schiff positive material comparable to those previously encountered only in shocked mice given dextran. Both casts and intracellular granules were present in this specimen. Liver of this particular mouse was unusually deficient in water soluble periodic-Schiff positive material.

DISCUSSION

Tissue sections of kidney and liver from mice given intravenous dextran were found to contain a material that, like dextran, was freely soluble in water but insoluble in alcohol. Like dextran, the material present in tissue sections of dextran-treated mice was found to be periodic acid-Schiff positive. To prevent solution of the dextran-like material during periodic-Schiff staining, it was necessary to use alcoholic instead of aqueous reagents. In sections of alcohol-fixed tissue cut without water flotation and stained by the alcoholic periodic-Schiff method, the dextran-like material was sharply localized and colored deep purple; it was present in blood vessels, parenchymal cells, and renal tubules. Tissues from a total of thirty-one dextran-treated and nineteen mice receiving no dextran were examined for the dextran-like material; it was present in all dextran-injected mice but absent in all of the controls. Hence, the possibility that the dextran-like material is some naturally occurring substance appears unlikely. The material shown is not believed to be glucose because the latter is alcohol soluble and not stained by our method. The coarsely globular and homogeneous nature of the deposits, the free solubility in water, and the pattern of distribution make it most unlikely that the dextran-like material is glycogen. Until some chemically different metabolic product of infused dextran can be shown to resemble dextran in solubility and periodic-Schiff reaction, it seems justifiable to regard the material demonstrated as incompletely hydrolyzed dextran.

The finding of material presumed to be dextran in the lumen of blood vessels was expected, and deserves no special comment. However, the finding of dextran inside liver and renal tubular cells is of considerable interest. The mechanism by which dextran enters the cell is not established. Opinions concerning the effects and the ultimate fate of intracellular dextran must await further study.

As dextran is known to be excreted in part by the kidneys, its occurrence in the lumina of renal tubules cannot be considered remarkable. What may be of more interest are the differences in dextran content and distribution in kidneys of shocked versus normal mice. All of sixteen mice given dextran after burn shock showed abundant casts and intracellular deposits of dextran in renal tubules. Of fifteen normal mice given the same or larger dose of dextran, comparable deposits were observed in only one. All of the animals in these experiments were sacrificed five hours after dextran injection. Therefore, the idea that greater amounts of dextran tend to enter the renal tubules of shocked mice must be regarded as only tentative; kidneys of both shocked and normal mice must be compared at various time intervals after dextran injection.

The alcoholic periodic-Schiff method should prove useful in the histochemical study of other water-soluble, alcohol-insoluble substances. Periodic-Schiff spot testing of such substances is considerably simplified since one can work with insoluble residues instead of solutions. Hotchkiss earlier suggested the use of periodic acid in seventy per cent alcohol for staining water soluble

substances but did not specify that paraffin blocks be cut in any special manner.[9] The alcoholic periodic-Schiff method presented differs from the Hotchkiss technique in the use of alcoholic reagents throughout the various steps in the reaction and in the omission of a reducing rinse prior to Schiff reagent. The use of alcoholic reagents throughout periodic-Schiff staining favors the conservation of water soluble, alcohol insoluble material.

SUMMARY

In sections of liver and kidney of normal mice given dextran intravenously and sacrificed five hours later, a material was regularly found that resembled dextran in both solubility and periodic-Schiff reaction. To prevent loss of the material during processing, tissues were fixed in alcohol and paraffin sections floated on alcohol instead of water. Sections were stained by a modified periodic-Schiff method using reagents prepared in seventy per cent alcohol instead of water. Material believed to be dextran was demonstrated in blood vessels, liver cells, and sometimes in renal tubules. Similar material was never observed in identically treated tissues of mice not given dextran.

Kidneys of mice receiving the same dose of dextran after experimental burn shock and sacrificed five hours later showed much more dextran in tissue sections. The additional dextran occurred largely as homogeneous masses filling many of the distal convoluted and collecting tubules and as cytoplasmic granules in proximal convoluted tubules.

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THE NEPHROTIC SYNDROME IN RATS; WITH SPECIAL REFERENCE TO THE PROGRESSION OF THE GLOMERULAR LESION AND TO THE USE OF NEPHROTOXIC SERA OBTAINED FROM DUCKS

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A RENAL disease simulating the nephrotic syndrome of children can be induced in rats by the intravenous injection of nephrotoxic sera obtained from rabbits.[1] In the disease of children and in this experimental disease of rats there may or may not be visible histologic alteration of the glomeruli. The prognosis of the experimental disease in rats, as well as of the spontaneous disease of children, depends chiefly on the progression of the glomerular injury. This communication deals with some variable factors that might influence the progression of the glomerular lesion in the rat. These factors include: (a) the strain of rats used, (b) the effect of aging of the sera, and (c) the species of the antibody-producing animal.

METHODS

The experimental procedures used in producing the nephrotoxic disease have been described previously.[1] Rats of the Long-Evans and Whelan* strain were used. Full-grown Muscovy ducks were treated with intraperitoneal injections of rat kidney extracts according to the procedures described for rabbits. They were bled according to the same schedules, and their sera were treated in the same fashion. The urinary proteins were quantitatively determined by the method of Shevky-Stafford.[2] The urine was collected for sixteen to twenty hours, and the values were corrected for twenty-four hour periods. All other methods used were the same as previously described.

RESULTS

Importance of the Strain of Rats.—The evaluation of 338 rats of the Long-Evans strain and 156 rats of the Whelan strain (Table I) shows that the sus-

TABLE I

	RABBIT SERUM				DUCK SERUM	
	LONG-EVANS STRAIN		WHELAN STRAIN		LONG-EVANS STRAIN	
	NO.	%	NO.	%	NO.	%
Number of animals	338		156		31	
Spontaneous cures	77	22.7	36	23.0	1	3.2
Died within 2 weeks	114	33.8	77	49.4	2	6.5
Anasarca	157	46.5	95	61.0	9	2.9
Microscopic hematuria	138	41.0	101	65.0	26	84.1
Hypertension	76	38.4	66	70.0	28	93.3
Uremia	11	3.25	17	10.9	16	51.6
Histologic glomerular lesions at onset		72.0		73.0		
Latent period	0	0	0	0	28	90.0

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ceptibility of these two strains to heteronephrotoxins obtained from rabbits does not differ appreciably. The sera were used in the same doses in rats of the same age which were fed the same rations, and the incidence and the severity of the renal disease at the time of onset did not vary significantly. The incidence of diffuse glomerular lesions noted histologically within the first three weeks of a severe disease was almost identical in the two groups. It was noted, however, that slight to moderate proliferation of cells and fibrils in the glomerular capsule, with adhesions to the tuft, were frequently noted in the first week of the disease in rats of the Whelan strain. This was rarely seen in rats of the Long-Evans strain. During the subsequent course of the disease a greater incidence of hematuria, hypertension and uremia occurred in rats of the Whelan strain (Table I). In Long-Evans rats with renal disease for six to fifteen months, the average B.U.N. and blood creatinine values were 34 and 1.35 mg. per cent, respectively. The corresponding values in Whelan rats were 67 and 2.1 mg. per cent. Diffuse proliferative glomerular lesions in these late stages of the disease were usually more marked and more frequent in Whelan than in Long-Evans rats.

Effect of Aging of the Sera.—The 235 rats observed from April, 1947, to January, 1949, listed in the left column of Table II represent the material on which our first publication[1] was based. It was noted that during the following twenty-two months the incidence of hypertension, hematuria, and uremia rose, and the incidence of spontaneous cures decreased in a series of 103 rats under observation from January, 1949, to November, 1950 (Table II). All

TABLE II

STRAIN	LONG-EVANS	LONG-EVANS
Number of rats	235	103
Period of observation	April, 1947–Jan., 1949	Jan., 1949–Nov., 1950
Died within first 2 weeks	33 per cent	36 per cent
Edema and ascites	35 per cent	70 per cent
Spontaneous cures	30 per cent	7 per cent
Microscopic hematuria	33 per cent	60 per cent
Hypertension	27 per cent	59 per cent
Uremia	1.7 per cent	7 per cent

experimental procedures were equal for both groups, except for the age of the sera used. From the day the sera were obtained from treated rabbits until the day the rats were injected the sera were kept frozen. The incidence of anasarca, hematuria, hypertension, and uremia rose, and the incidence of spontaneous cures was strikingly smaller in the group of rats treated with sera that had been kept frozen for as long as three and one-half years. The sera were kept in 5 ml. viols, and were subjected to thawing and refreezing in accordance with the frequency with which they were used.

The Effect of Rat Anti-kidney Sera Obtained from Ducks.—When rats of the Long-Evans strain were injected with nephrotoxic sera obtained from ducks instead of rabbits (Table I), two striking differences in the course of the disease became apparent. A latent period was noted in 90 per cent of the rats, and the incidence of progressive azotemia, hypertension, and microscopic hematuria was much higher.

The latent period, varying from three to twenty-one days, was observed in twenty-eight of thirty-one rats. Fig. 1 shows the excretion of urinary proteins in milligrams per twenty-four hours in three rats injected with the same dose of a nephrotoxic duck serum. The latent period was three days in two rats and six days in the third. The amount of proteins excreted in the urine up to the third and sixth days, respectively, was within the range of the physiologic proteinuria characteristic of this strain. Gross hematuria was absent, but the benzidine reaction was intermittently positive in all three animals. Nevertheless, many specimens had a negative benzidine test in spite of massive proteinuria. A fluctuating, moderate to marked hypertension was noted in these, as well as in 93 per cent of all rats injected with duck serum. The in-

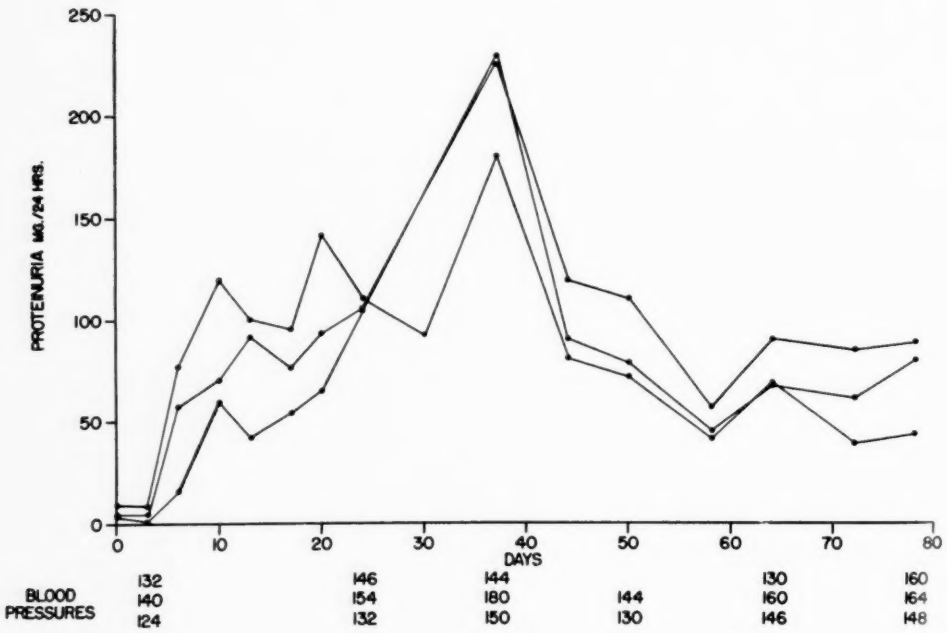

Fig. 1.

cidence of spontaneous cures was definitely lower than in rats injected with nephrotoxic rabbit sera (Table I). By virtue of the slow start due to the latent period fewer rats died within the first three weeks or developed marked ascites and edema when injected with sera obtained from ducks (Table I).

A comparison of the blood chemistry changes that were noted in different stages of the disease in rats injected with duck and rabbit sera is shown in Fig. 2. It can be seen that azotemia was more marked in early phases of the disease in rats that were injected with rabbit sera. As the disease progressed, however, the azotemia decreased. In rats injected with duck sera, the azotemia was milder in the early stages but increased as the disease went on. A similarly progressive increase of the blood creatinine values (even though

less marked) was noted in animals that received duck sera. The serum protein concentration showed similar degrees of hypoproteinemia in both groups through all stages. Nephrotic hyperlipemia, with elevation of blood cholesterol, phospholipids, and total lipids, was present in rats treated with duck sera as regularly as it had previously been noted when rabbit sera were used. The only difference of note was that the cholesterolemia was more marked in early phases of the disease in rats that received sera obtained from rabbits.

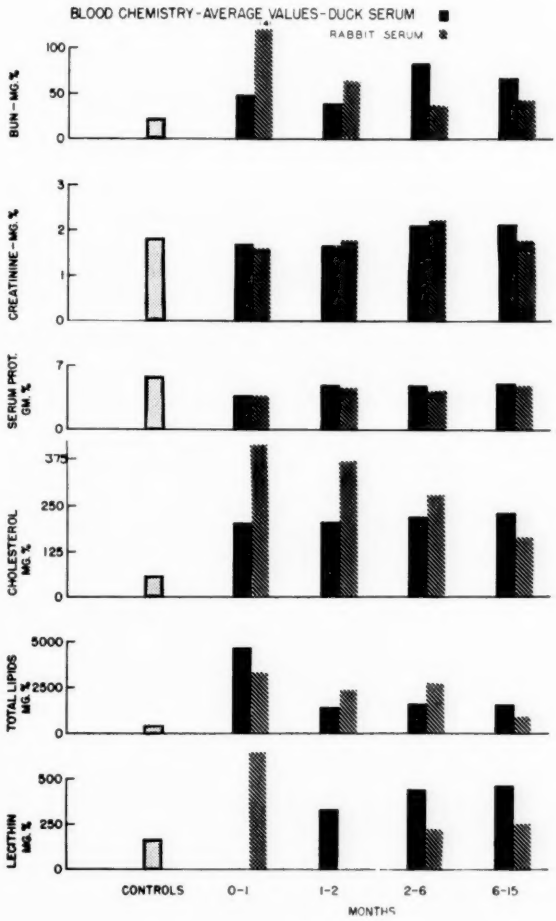

Fig. 2.

Hisologic studies were carried out in eighteen of the thirty-one rats injected with the duck nephrotoxin. The glomerular, tubular, and interstitial tissue changes were similar to those observed in animals injected with rabbit serum. The changes were definite in fifteen rats, and were focal and of only a slight degree in three rats. In the early stages there was thickening of the capillary basement membrane, which was well demonstrated by the periodic acid-Schiff staining technique. There was also some endothelial proliferation

and a marked decrease in the vascularity of the glomerular tufts. In later stages there was marked fibrosis of almost all the glomeruli, and diffuse interstitial infiltration by chronic inflammatory cells. Tubular changes included hyaline casts in the lumen, and hyaline droplet changes and fatty degeneration of the lining cells. These tubular changes roughly paralleled the glomerular changes in degree of severity.

DISCUSSION

Smadel and Swift[3] have described differences in the response of various strains of rats to the injection of nephrotoxic sera which are essentially in agreement with our results. They believed that Whelan rats were possibly slightly more susceptible to heteronephrotoxins and noted that "the number of completely fibrosed glomeruli and the amount of crescent formation were slightly greater in the Whelan rats." We have not noted that rats of the Whelan strain were more susceptible than Long-Evans rats to develop the initial injury. Smadel and Swift were not convinced that the difference in susceptibility which they described was significant. In our studies the dose of nephrotoxin was gauged according to kidney weight and not according to body weight as in Smadel's and Swift's work. Consequently, rats weighing less than 300 Gm. obtained higher doses in our studies, to an extent that the amount of sera that we used in 4- to 5-week-old animals was twice as large. It is possible that the larger doses administered to our animals accounts for the discrepancy between our findings and those of Smadel and Swift. The incidence of glomerular lesions at onset was the same in both strains, indicating that the decreased ability of Whelan rats to recover was not due to a higher incidence of initial glomerular involvement. The increased occurrence of azotemia, hematuria, and hypertension in rats of the Whelan strain thus is probably due to a greater tendency of the glomerular injury to progress to fibrosis and hyalinization.

The observation of a similar effect of sera that were kept frozen for one and one-half to three and one-half years, to enhance the progression of the glomerular lesions to irreversible fibrosis, needs further verification. The high incidence of marked anasarca and the frequent necessity to reduce the dose of older sera suggests that the nephrotoxicity of these sera increased with aging. More work will have to be done, however, to clarify these observations. They would suggest that the chemical or physico-chemical state of the antibody is, aside from its concentration, of importance for the specific nature of the injury that it produces in the kidneys.

The importance of the chemical structure of the nephrotoxin for the progression of the glomerular lesion is also shown by using sera obtained from ducks. The same strain of rats that had an incidence of uremia of 3.25 per cent when sera obtained from rabbits were injected, showed an incidence of 52 per cent when duck sera were used. These observations were confirmed by the histological studies of the kidneys. In the later stages of the disease there was more severe fibrosis of the glomeruli following the use of duck sera than following the use of rabbit sera.

A latent period was noted in rats injected with duck sera, which had not been observed when rabbit sera were used. Pearce,[4] who used dog antikidney sera obtained from rabbits, did not mention the observation of a latent period. The renal disease produced in rabbits following an injection of nephrotoxic serum from ducks is known to occur after a latent period of five to ten days.[5, 6] It is therefore likely that the latent period is due to some characteristic of the nephrotoxic duck serum, rather than to a difference in response of the rat and rabbit. Fouts, Corcoran, and Page[7] have shown that a latent period of six to ten days occurs in dogs injected with nephrotoxic serum prepared in the hen. It may be inferred from this that the latent period is due to some characteristic of fowl serum.

Aside from these differences the renal disease produced in rats by duck sera simulated the nephrotic syndrome as it was observed when rabbit sera were used. Hypoproteinemia and marked hyperlipemia, with increase in cholesterol, phospholipids and total lipids, were regularly noted in direct relation to the severity of the disease. Anasarca was less frequently noted with duck sera which may be due to the slower rate with which proteinuria sets in. Since the etiology of the nephrotic syndrome in infants and children is not clear, it is not known whether a latent period is part of the human disease. The eliciting mechanism is unclear, the onset is insidious, and a latent period thus may escape detection.

As far as the progression of the glomerular lesion is concerned, great variations do exist in the disease that afflicts children. The benign course, that may terminate in recovery in many of these patients, is more reminiscent of the disease that is produced in 4- to 5-week-old rats of the Long-Evans strain with freshly obtained rabbit sera. The occasional death of children, within one or more years, from renal failure associated with azotemia, creatinine retention, hypertension, and renal rickets is more in keeping with the observations made on rats of the Whelan strain injected with rabbit sera or of either Long-Evans or Whelan rats treated with nephrotoxic sera obtained from ducks.

SUMMARY

Antirat kidney sera obtained from rabbits were used in 338 rats of the Long-Evans and 156 rats of the Whelan strain. The susceptibility of these two strains to heteronephrotoxins did not differ. The incidence of diffuse glomerular lesions during early stages of a severe disease was almost identical. Diffuse proliferative glomerular lesions in late stages, however, were more marked and more frequently noted in Whelan than in Long-Evans rats. The incidence of uremia was 3.25 per cent in the Long-Evans and 10.9 per cent in the Whelan rats.

One hundred three Long-Evans rats were injected with nephrotoxic rabbit sera that were kept frozen for one to three and one-half years, and 235 were injected with sera which were less than one year old. The group given the older sera showed a greater incidence of anasarca, hematuria, hypertension, and uremia, and a smaller incidence of spontaneous cures.

Thirty-one Long-Evans rats were injected with nephrotoxic sera obtained from ducks. A latent period varying from three to twenty-one days was noted in twenty-eight of the thirty-one animals. No latent period was present when rabbit sera were used.

The renal disease produced by the duck sera differed from that produced by rabbit sera in being milder at the onset, but with a greater tendency to progression. This resulted in a more severe chronic disease with a greater incidence of hypertension and uremia. Hypoproteinemia and hyperlipemia were regularly observed in both groups.

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THE SERUM IRON IN ACUTE HEPATITIS

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THE European literature has for several years contained references to the alterations of the serum iron in infectious hepatitis, but no published data on the subject have been accumulated in this country. Hemmler (1939)[1, 2] first studied the serum iron in cases of jaundice. He observed that in obstructive jaundice (i.e., common duct stones and carcinoma of the head of the pancreas) the values were within normal limits, but in catarrhal icterus (infectious hepatitis) there was a definite elevation in the serum iron. In 1927 Warburg and Krebs,[3] and in 1932, Locke and associates[4] each reported a single case of "icterus" (hepatitis?) with hypersideremia. Since Hemmler's report in 1939, several references to the alterations in the serum iron in acute hepatitis have appeared in the European literature.[5-11] There are also publications by Skouge,[12] and Vannotti and Delachaux,[13] that refer to the hypersideremia in acute hepatitis.

It is the purpose of this paper to present the results of serial serum iron determinations in sixty cases of acute viral hepatitis.

MATERIALS AND METHODS

The sixty cases of acute viral hepatitis represent forty-seven cases of presumably homologous serum viral hepatitis (all but nine in battle casualties from Korea), and thirteen cases of sporadic, endemic viral hepatitis. The majority of the patients were young adult men. The group included six women patients. With but few exceptions (because of technical difficulties involved in collecting blood) these patients represent sixty consecutive cases of acute hepatitis admitted to the Hepatic and Metabolic Disease unit, Walter Reed Army Hospital, from Nov., 1950, to July, 1951. The diagnosis of acute hepatitis was confirmed by liver biopsy in thirty-five of the cases. An average of five to eight separate determinations for serum iron was performed throughout the course of the disease, or a total of 340 separate determinations in the sixty cases.

The serum iron determinations were performed by a modification of the method of Kitzes and associates,[14] Barkan and Walker,[15] and Fortune and Mellon.[16]

The serum bilirubin was performed by the Ducci and Watson[17] modification of the method of Malloy and Evelyn,[18] the thymol turbidity by the method of MacLagan,[19] the cephalin-cholesterol flocculation by the method of Hanger,[20] and the gamma globulin turbidities by the method of Kunkel[21] and de la Huerga and Popper.[22] Cholinesterase determinations were performed by the method of Michel,[23] urine bilirubin by a modification of the Harrison spot test,[24] and bromsulfalein retention by the method of Gaebler.[25]

RESULTS

Fig. 1 shows the results of the early morning serum iron values in fifty young normal individuals (fifteen women and 35 men), in the sixty cases of acute hepatitis (only the highest recorded value), and in various other liver

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and biliary tract diseases. The mean for the normals was 130 γ per 100 ml. and for the cases of hepatitis, 295 γ per 100 milliliters. For the cases of obstructive jaundice caused by common duct stone and carcinoma of the head of the pancreas, the mean was 155 γ per 100 ml. (ten cases). The mean for the fifteen cases of "portal" cirrhosis was 110 γ per 100 ml., for "chronic hepatitis," 180 γ per 100 ml. (twelve cases), and "fatty" liver, 140 γ per milliliter (four cases). In the cases of "fatty" liver "chronic" hepatitis, and in all but one case of cirrhosis the diagnosis was made with the aid of liver biopsy.

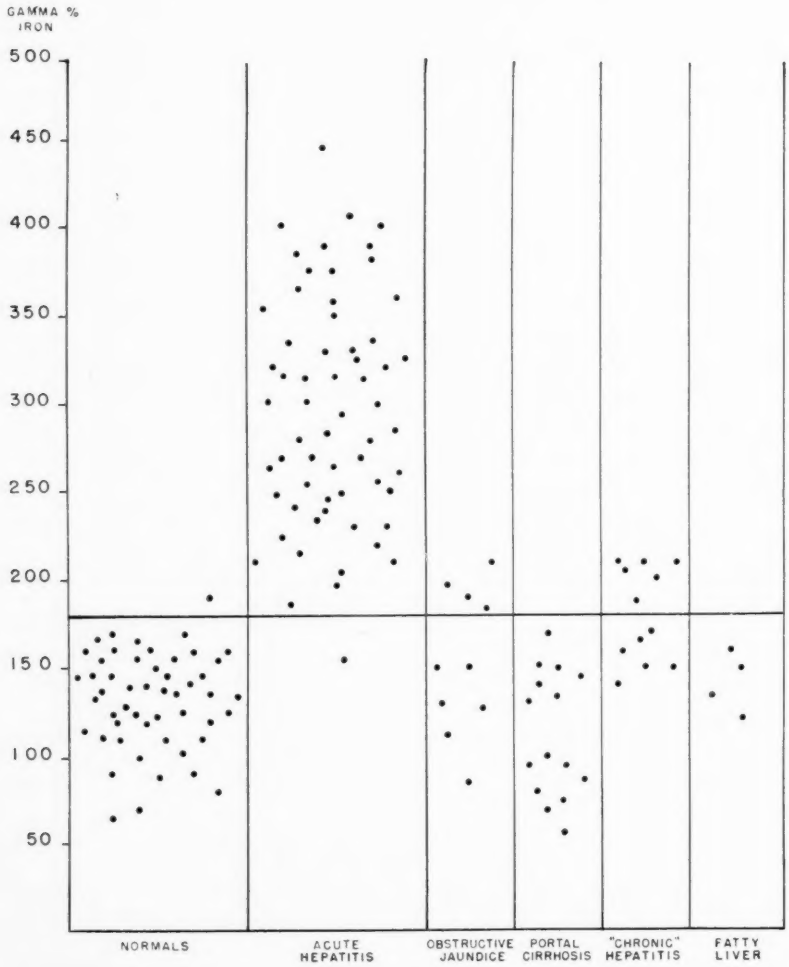

Fig. 1.—The serum iron in normal individuals, and in patients with various liver and biliary tract diseases. For the latter group only the highest recorded value for each patient is listed.

In the sixty cases of acute hepatitis fifty-five of the cases, or 92 per cent, had a serum iron value of over 200 γ per 100 ml. at some time during the first fifteen days after the onset of clinical jaundice. There were five patients that

did not show an elevation above 200 γ per 100 ml. at any time throughout the course of their disease. Three of these cases were of mild severity and had a maximum bilirubin value of less than 5.0 mg. per 100 ml. (28 per cent of the sixty patients had maximum bilirubin values below 5.0 mg. per 100 ml.). Of the other 2 patients, one had a maximum bilirubin value of 8.0 mg. per 100 ml., and the other patient had a maximum value of over 20 mg. per ml. This latter patient, however, had a moderately severe chronic infection (cellulitis) in association with his hepatitis, with a fever of 101-103° F. and a leukocytosis of 20,000 to 25,000.

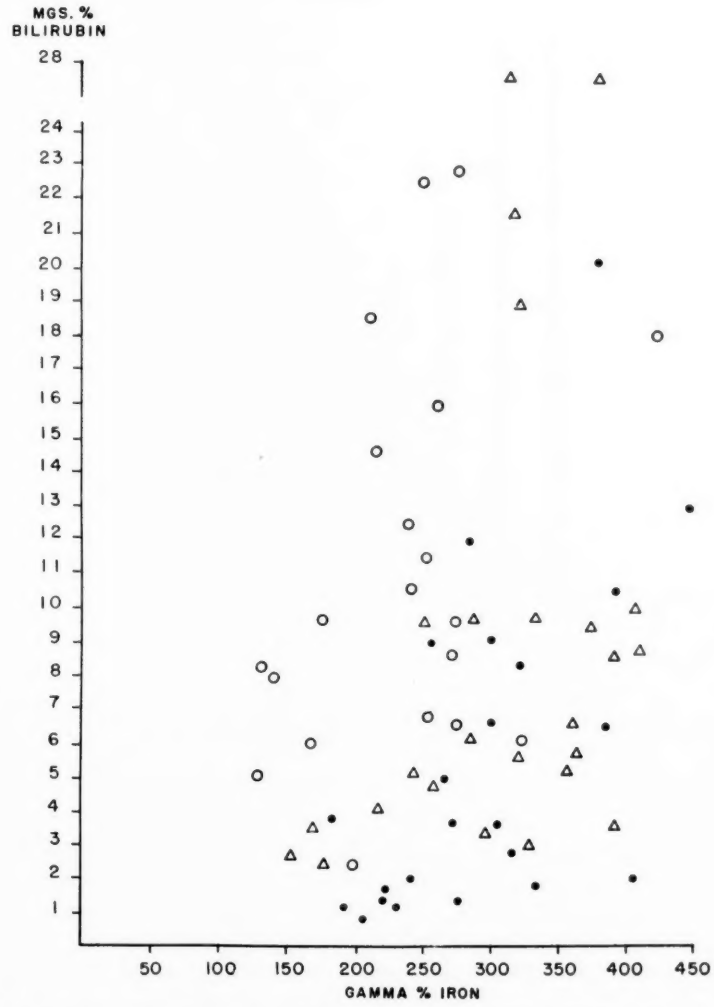

Fig. 2.—The relationship of the serum iron and serum bilirubin. The circles represent the maximum level of serum bilirubin obtained in individual cases of hepatitis and the level of the serum iron on the same day. The dots represent the maximum level of the serum iron obtained in individual cases and the level of the serum bilirubin obtained on the same day. The triangles indicate periods when the maximum elevation of the serum bilirubin and the serum iron were obtained on the same day.

Of the five patients who did not show an abnormally elevated serum iron, four were serum viral hepatitis and one was endemic viral hepatitis. Of these five patients, three received intravenous or intramuscular ACTH early in the course of their disease. In the other patients receiving ACTH (twenty-seven patients) there appeared to be no striking correlation between the serum iron levels and administration of ACTH.

Two patients showed a mild clinical and chemical relapse, and, coincidental with this relapse, a slight return rise in the serum iron.

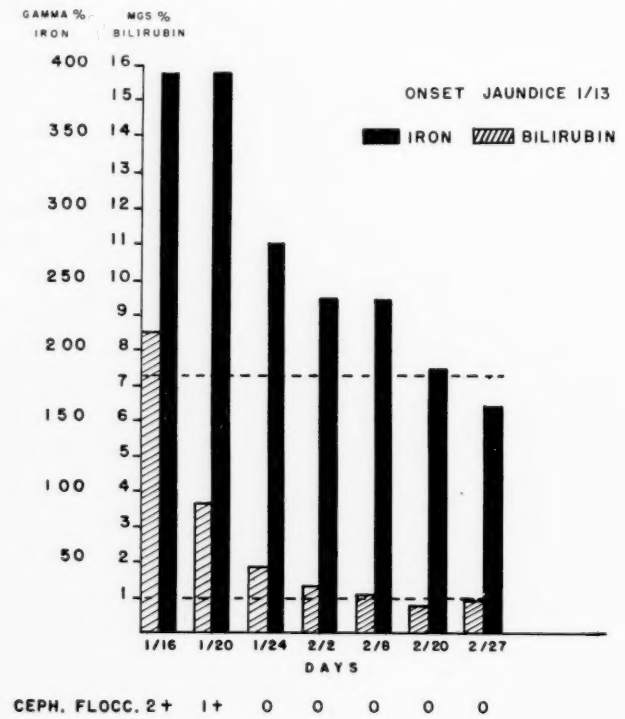

Fig. 3A.—The course of the serum iron, the serum bilirubin, and the cephalin cholesterol flocculation test in a representative case of acute hepatitis.

When the serum iron is compared with other liver function tests (serum bilirubin, cholinesterase, and the flocculation tests) there is no clear-cut correlation. However, the more severe the case and the more abnormal the liver function generally, the higher the serum iron. Table I lists the serial liver function tests and the serum iron in twelve representative cases of the 60 studied.

The maximal hypersideremia and the maximal serum bilirubin level may occur at the same time; however, in more than 40 per cent of the cases the maximal serum iron did not occur until after the serum bilirubin had begun to fall. The maximal serum bilirubin occurred at three to four days after the onset of clinical icterus and the maximum serum iron occurred at five to six

days after the onset of clinical icterus (average of the 60 cases). Fig. 2 is a scattergram which shows this lack of correlation between the serum iron and the serum bilirubin. Fig. 3 shows graphically the course of the serum iron and the serum bilirubin in four of the cases of acute hepatitis (including one case that received intravenous ACTH). In general, the serum iron and serum bilirubin returned to normal levels at about the same time. The serum iron returned to a level of less than 200 γ per 100 ml. at approximately thirty-seven days after the onset of clinical icterus.

Fig. 4 shows a comparison of the serum iron with other commonly used liver functions tests in acute hepatitis.

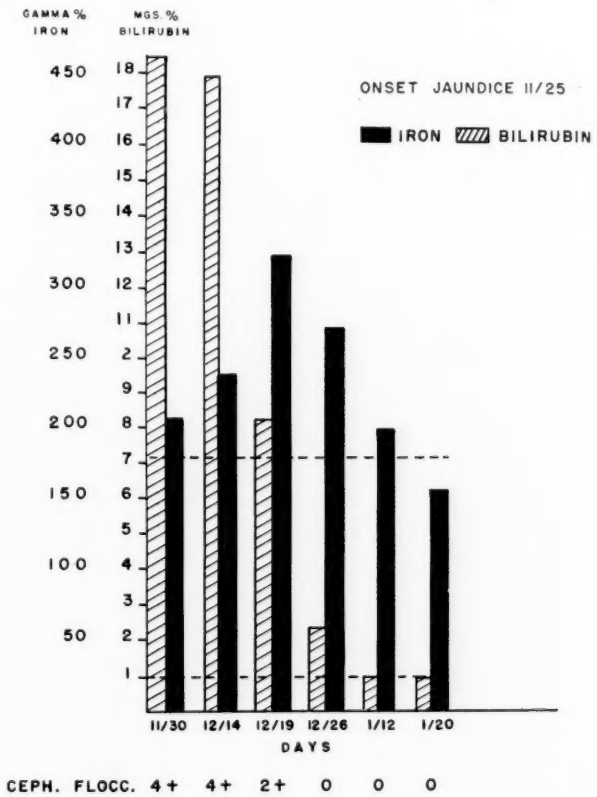

Fig. 3*B*.—The course of serum iron, the serum bilirubin, and the cephalin cholesterol flocculation test in a representative case of acute hepatitis.

DISCUSSION

The serum iron makes up only about 0.1 per cent of the total body iron.[26] However, it by no means exists as a static component in the serum. The serum iron is completely turned over about ten times every twenty-four hours (about 50 per cent every two hours); 25 to 30 mg. per day.[27] Thus, any marked disturbances in iron utilization, storage, or transport, are likely to be associated

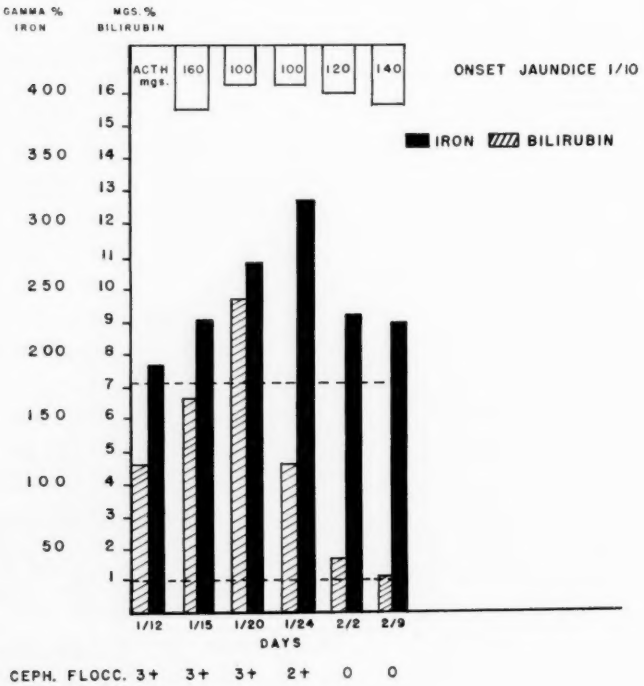

Fig. 3C.

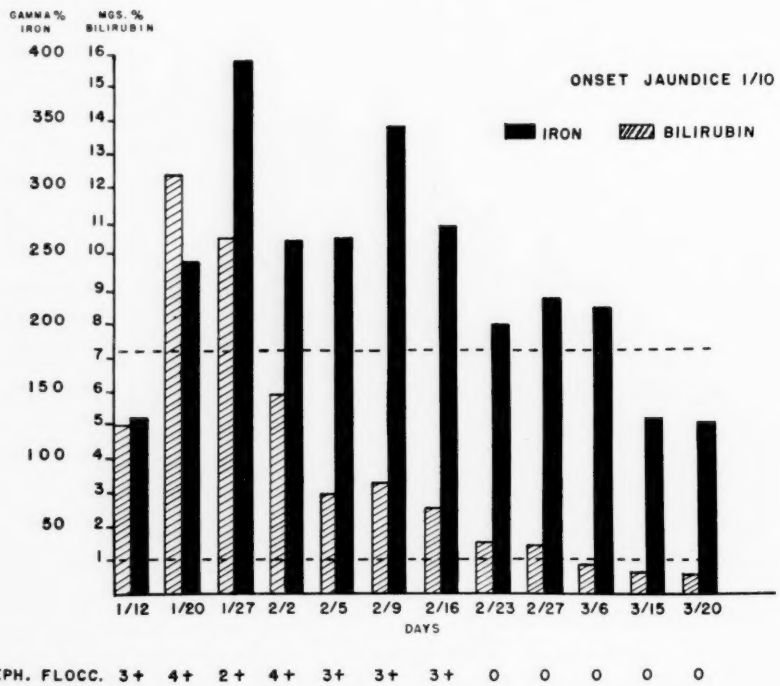

Fig. 3D.

Figs. 3C and 3D.—The course of serum iron, the serum bilirubin, and the cephalin cholesterol flocculation test in two representative cases of acute hepatitis.

with changes in the level of the serum iron. Also, since the liver contains approximately 15 per cent of the total body iron,[26, 28] it is not surprising that an injury to the liver might affect the storage and transport of iron.

There are few diseases other than acute hepatitis that may produce an elevation in the level of the serum iron, viz., increased red cell hemolysis,[13] aplastic anemia,[13, 29] pernicious anemia,[29, 30] and hemochromatosis.[31] In these conditions the serum iron is usually only slightly elevated. Thus, acute hepatitis is the only disease in human beings which is consistently associated with a marked hypersideremia.

In some respects, the hyperideremia of acute hepatitis is an unexpected finding, because in most infectious or inflammatory diseases the serum iron is low or low normal.[7, 32, 33] One patient in the present series with a moderately severe infection associated with a moderately severe hepatitis, failed to show a hypersideremia at any time during the course of his disease.

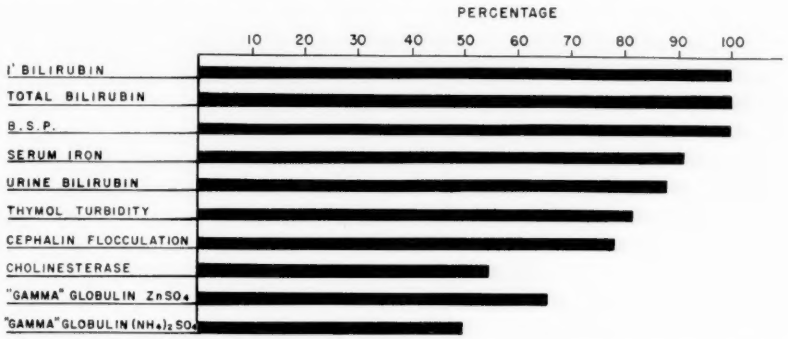

Fig. 4.—The percentage of times that the serum iron and various routine liver function tests were found to be significantly abnormal at some time during the first fifteen days of clinical icterus, in sixty cases of acute hepatitis.

Acute hepatitis also seems to present a unique picture with respect to the liver and iron metabolism. In portal cirrhosis, which in many instances probably represents a more severe degree of liver injury (hepatocellular damage), there is no hypersideremia.[8-11] In one of the patients studied, with a severe cirrhosis and ascites, who had a serum bilirubin of 18.0 mg. per 100 ml., the serum iron never rose higher than 160 γ per 100 ml. Two patients with cirrhosis and hepatic coma had persistently normal serum iron levels.

Hyperbiiirubinemia is not necessarily associated with hypersideremia. In jaundice associated with cirrhosis the serum iron is within normal limits. In jaundice, due to obstruction of the common bile duct, by either a stone or malignancy, there is no hypersideremia.[1, 2, 8, 10, 11] One case of obstructive jaundice due to a common duct stone showed a serum bilirubin of 12.5 mg. per 100 ml., with a serum iron of 195 γ per 100 mg. Another case with a carcinoma of the head of the pancreas had a serum bilirubin of 25.0 mg. per 100 ml., and a serum iron of only 150 γ per 100 ml.

Also, in acute hepatitis there is no linear relation between the height of the serum bilirubin and the height of the serum iron. In many of the cases

TABLE I

CASE	FE	T.B.	CHE	T.T.	C.F.	B.U.	
*No. 1, HSH**							
30 Nov.	210	18.5	0.50	8.1	4+	4+	Onset icterus Nov. 25
4 Dec.	240	17.9	0.61	8.6	4+	4+	
9 Dec.	320	8.4	0.57	5.4	2+	2+	
26 Dec.	270	2.3	0.95	3.7	0	-	
12 Dec.	200	1.5	–	3.6	0	-	
20 Dec.	160	1.0	–	4.4	0	-	
No. 2, HSH†*							
6 Dec.	275	6.4	0.52	5.7	3+	4+	Onset icterus Dec. 6
11 Dec.	305	3.7	0.56	5.0	2+	0	
19 Dec.	270	1.9	0.74	3.2	1+	0	
29 Dec.	250	1.5	0.77	2.0	2+	0	
5 Jan.	230	1.4	0.84	2.0	0	–	
10 Jan.	220	1.3	–	1.5	0	–	
20 Jan.	270	1.7	0.80	1.6	0	–	
24 Jan.	170	0.6	0.73	1.6	1+	–	
2 Feb.	250	1.2	0.74	1.0	0	0	
16 Feb.	230	1.0	0.74	1.0	0	0	
23 Feb.	150	0.5	0.74	1.0	0	0	
6 Mar.	185	1.8	0.74	1.1	0	0	
No. 3, HSH†*							
18 Dec.	175	2.4	0.62	5.9	4+	0	Onset icterus Dec. 8
29 Dec.	175	1.5	0.72	3.6	1+	0	
10 Dec.	150	1.1	0.95	3.0	0	0	
28 Dec.	175	0.8	0.86	1.4	0	–	
No. 4, HSH							
10 Jan.	275	22.8	0.83	6.3	4+	4+	Onset icterus Dec. 25
20 Jan.	385	6.6	0.34	3.7	3+	0	
24 Jan.	350	5.1	0.81	3.4	1+	0	
9 Jan.	260	3.0	–	2.9	1+	0	
16 Jan.	230	1.5	–	2.5	2+	0	
23 Jan.	195	1.2	0.80	2.3	0	0	
27 Jan.	125	1.0	0.95	2.2	0	0	
*No. 5, HSH**							
12 Jan.	130	5.0	0.68	5.4	3+	1+	Onset icterus Jan. 10
20 Jan.	240	12.4	0.58	7.5	4+	–	
27 Jan.	390	10.5	0.55	7.5	2+	1+	
2 Feb.	265	5.8	0.67	6.5	4+	0	
5 Feb.	265	3.0	–	5.9	3+	0	
9 Feb.	340	3.2	–	5.2	3+	0	
16 Feb.	270	2.5	0.81	5.9	3+	0	
23 Feb.	195	1.5	0.78	3.9	0	0	
27 Feb.	215	1.3	0.89	3.3	0	0	
6 Mar.	210	0.9	1.00	3.3	0	0	
15 Mar.	130	0.6	0.95	3.3	0	0	
20 Mar.	125	0.5	1.00	2.0	0	–	
No. 6, HSH†*							
12 Jan.	190	4.7	0.68	3.6	3+	–	Onset icterus Jan. 10
16 Jan.	230	6.7	0.53	7.7	3+	–	
20 Jan.	275	9.6	0.58	7.3	3+	–	
24 Jan.	315	2.6	0.68	4.7	1+	–	
2 Feb.	230	1.6	0.83	2.9	0	–	
9 Feb.	225	1.1	0.85	2.5	0	–	
*No. 7, HSH**							
16 Jan.	390	8.5	0.88	2.9	2+	–	Onset icterus Jan. 13
20 Jan.	390	3.6	0.86	2.5	1+	–	
24 Jan.	275	1.8	0.89	2.3	1+	–	
2 Feb.	235	1.3	–	2.1	0	0	

the maximal sideremia occurred after the bilirubin had begun to fall. Also the serum iron often persists at a high level while the bilirubin is declining. Other investigators have made this same observation.[1, 2, 6, 11]

TABLE I—CONT'D

CASE	FE	T.B.	CHE	T.T.	C.F.	B.U.	
No. 8, IH†*							
23 Feb.	420	18.0	0.39	7.9	4+	–	Onset icterus Feb. 19
2 Mar.	445	13.0	0.44	7.5	3+	–	
9 Mar.	310	9.0	0.40	6.5	3+	–	
15 Mar.	270	5.5	0.53	4.7	3+	–	
20 Mar.	200	3.3	0.54	4.0	0	–	
23 Mar.	200	3.3	0.62	3.2	0	–	
29 Mar.	190	2.3	0.60	3.0	0	–	
3 Apr.	190	1.9	0.69	3.6	0	–	
13 Apr.	175	0.9	0.70	4.4	0	–	
No. 9, IH							
16 Apr.	300	4.2	1.30	2.1	0	3+	Onset icterus April 12
21 Apr.	335	9.6	1.20	4.2	1+	4+	
2 May	240	2.2	1.40	2.7	0	1+	
5 May	245	1.9	1.60	2.6	0	0	
8 May	210	1.6	1.50	2.5	0	–	
11 May	200	–	–	–	–	–	
15 May	170	1.1	1.70	2.7	0	–	
24 May	175	0.9	2.20	2.0	0	–	
6 June	170	0.8	–	–	–	–	
No. 10, HSH							
25 Apr.	325	21.5	0.67	6.1	4+	4+	Onset icterus April 23
30 Apr.	215	13.1	0.61	11.6	4+	4+	
2 May	285	7.3	0.78	9.8	4+	1+	
5 May	210	2.4	1.00	10.4	4+	0	
16 May	205	2.3	1.50	3.6	2+	0	
21 May	190	1.6	1.70	3.2	3+	0	
28 May	180	1.2	1.50	2.6	–	0	
11 June	130	0.7	1.40	2.3	0	–	
No. 11, HSH							
27 Apr.	315	5.7	0.55	6.8	3+	2+	
2 May	200	4.7	0.48	5.9	4+	1+	Onset icterus April 23
9 May	175	2.1	0.58	5.7	4+	0	
11 May	185	2.0	0.65	4.2	3+	0	
16 May	150	1.5	0.85	4.7	3+	–	
No. 12, HSH							
8 June	200	1.2	1.00	4.0	4+	0	Onset icterus June 13
13 June	235	2.7	1.00	4.2	4+	0	
19 June	300	2.1	1.20	3.6	4+	0	
22 June	340	1.7	1.40	3.3	3+	0	
25 June	235	1.0	1.20	2.9	0	0	
6 July	205	0.9	–	2.0	0	0	
10 July	200	0.8	–	–	–	0	
17 July	180	0.8	–	–	–	0	

* = Liver biopsy section early in disease diagnosed as acute hepatitis.
† = Received intramuscularly and/or intravenously ACTH, early in course of disease.
HSH = Homologous serum hepatitis; IH, infectious hepatitis.
Fe = Serum Iron (gamma per 100 ml.): Normal, 70 – 180 gamma per 100 ml.
T.B. = Serum bilirubin (mg. per 100 ml.): Normal, 0.0 – 1.0 mg. per 100 ml.
Che. = Serum pseudocholinesterase (△ pH units): Normal, > 0.70 units.
T.T. = Thymol turbidity (units): Normal, 1 – 3 units.
C.F. = Caphalin-cholesterol flocculation (48 hr. reading): Normal, < 2 + 48 hr.
B.U. = Bilirubinuria.

As yet, no one has offered any convincing evidence which would help explain the hypersideremia that is seen in acute hepatitis. Some of the possible mechanisms that might explain the hypersideremia are: (1) a block to the deposition of transport iron in the inflamed liver, (2) increased mobilization of iron from the inflamed liver, (3) increased absorption of iron from the gastrointestinal tract, (4) decreased utilization of iron for hemoglobin synthe-

sis, (5) increased destruction of red blood cells, (6) decreased excretion of iron in the bile.

Vannotti and Delachaux,[13] on the basis of some experimental work in animals after phosphorous poisoning, have suggested that there may be a block to the deposition of transport iron in the inflamed liver. However, in cirrhosis, which may often represent a more severe degree of liver cell injury than found in hepatitis, there is no hypersideremia. Also, Vahlquist,[6] Skouge,[12] and Uhnoo[34] have shown that the oral and intravenous iron tolerance curves are the same in patients with acute hepatitis, and in normal individuals, except that the initial level is higher in the former.

There would have to be a tremendous increase in the gastrointestinal absorption, and in addition, a decreased deposition of iron in the liver if increased gastrointestinal absorption of iron were to explain the hypesideremia. Also, most all of the blood specimens in this series were drawn after a twelve- to fifteen-hour fast. If, because of the absence of bile in the gastrointestinal tract the body absorbed more iron, then a hypersideremia would probably occur in obstructive jaundice.

A decreased utilization of iron for hemoglobin synthesis would probably be associated with some degree of anemia, and this does not occur in hepatitis. Also, there is no evidence to indicate an increased destruction of red cells—there is no increase in reticulocytes.

Hemmler[1, 2] suggested that the hypersideremia might be due to a decreased excretion of iron through the bile, and that in conditions other than hepatitis, associated with a block to bile excretion (common duct obstruction), some other factor is involved which causes a decreased serum iron (cholangitis, malignancy). However, hepatitis like cholangitis, also represents an inflammation, and the experimental work on the excretion of iron would indicate that only a very insignificant amount of iron is excreted through the bile.[35, 36]

Thus, for the present, we are left without any satisfactory evidence that would explain the hypersideremia of acute hepatitis.

In view of the fact that the serum iron is so universally elevated in acute infectious hepatitis, it may have some value as an aid in differentiating between extrahepatic obstructive (surgical) jaundice, and jaundice due to acute hepatitis (but not the jaundice due to increased hemolysis). The serum iron level as a test of liver injury (i.e., cirrhosis) is not an extremely sensitive indicator, and probably would prove of no value in the differential diagnosis of the milder cases of jaundice. Also, data are not at present available on the serum iron in some of the other types of hepatitis—"pure" cholangiolitic hepatitis, toxic hepatitis, and hepatitis resulting from leptospira or amebic infestation.

When the serum iron determination is compared with other commonly used liver function tests it has been found to be more consistently positive (abnormal) in acute hepatitis than many of the tests, except for the determination of bromsulfalein retention.

The methods in use for the determination of the serum iron are sufficiently simple and short, providing special care is taken to use iron-free reagents and equipment, to make it useful as a special test (not as a routine determination) in certain cases of jaundice.

SUMMARY

Serial serum iron determinations were performed in sixty cases of acute infectious hepatitis, and various other cases of liver and biliary tract disease. The serum iron was found to be elevated to over 200 γ per 100 ml. in fifty-five of the sixty cases; range, 155 to 445 γ per 100 milliliters.

The serum iron was not found to be significantly elevated in cases of extrahepatic obstructive jaundice, cirrhosis, "chronic" hepatitis, or fatty infiltration of the liver.

In nearly one-half of the cases the maximal hypersideremia occurred a few days after the maximal bilirubinemia (maximal liver injury), and in most cases the hypersideremia persisted after the serum bilirubin had begun to fall.

The serum iron was more often positive (significantly abnormal) in acute hepatitis than most of the other commonly used liver function tests.

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THE INCIDENCE OF HEREDITARY HYPERCHOLESTEREMIA

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A DISTURBANCE of lipid metabolism is considered to be a predisposing factor in the genesis of certain forms of atherosclerosis. An elevation of the total serum cholesterol,[1] an alteration of the cholesterol : phospholipid ratio,[2] a high concentration of low density lipid and lipoprotein components in the serum as determined by ultracentrifugation,[3] and even the number and size of chylomicrons in the serum[4, 5] have been considered reflections of this disturbance. It is important to emphasize, however, that the precise role of each of these factors is still under investigation.

Of these disturbances of lipid metabolism, only that which is concerned with an elevation of total serum cholesterol has been shown to have any hereditary implications.[6a] Studies of this subject dealt only with single families,[6b-e] or with families of individuals known to have either xanthomatosis[7] or coronary artery disease.[8] An attempt was made, therefore, to estimate the incidence of hereditary hypercholesteremia in a larger and less selected population.

PROCEDURE

This study was conducted in a large general hospital in the metropolitan area of New York City. A total of 200 individuals, representing two series of 100 consecutive unselected admissions to a male and a female ward, were surveyed as index patients.

On admission a careful personal and family history was obtained from the patient, and a physical examination was performed with particular attention to the signs and symptoms of diseases associated with a disturbed lipid metabolism (corneal arcus, xanthelasma, xanthoma, and coronary artery disease). The morning after admission a specimen of venous blood was drawn after the patient had fasted for twelve hours. The serum cholesterol was determined by the Sperry-Schoenheimer method. Values of 280 mg. per 100 ml. or higher were considered to be hypercholesteremic.*

Whenever the history, the physical examination, or the total serum cholesterol of the index patient suggested the presence of hereditary hypercholesteremia, serum cholesterol determinations were performed on all available members of the immediate family. Hereditary hypercholesteremia was considered to be present whenever three or more immediate family members (parent, sibling, or child) were found to have a serum cholesterol level in excess of 280 mg. per 100 ml.

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*Using the Sperry-Schoenheimer method, 211 mg. per 100 ml. ±30, has been established as the normal total serum cholesterol value in our laboratory on the basis of 119 determinations on normal subjects. Cholesterol levels differed considerably in normal individuals; but in any single individual multiple cholesterol determinations over a period of six to eight weeks indicated that the level remained constant (±11 per cent). This is in agreement with reports of most of the recent investigators who have used the Sperry-Schoenheimer method for this determination.

RESULTS

This report presents the results obtained from the study of the 200 index patients, and the families of thirty-six of them, in whom hereditary hypercholesteremia was suspected. The age of the men in the index group ranged from 18 to 79 years, average 48.4 years. The women ranged in age from 12 to 80 years, average 46.5 years. The cholesterol level of the men varied from 111 to 512 mg., average 226 mg. per 100 ml. Among the women there was a range from 109 to 655 mg., average 241 mg. per 100 ml. The basic diseases of these patients were as follows: cardiovascular-renal, 40 men, 38 women; infectious, 18 men, 15 women; neoplastic 10 men and 6 women; endocrine, 3 men, 11 women; other diseases 29 men and 30 women.

Of the 200 index patients a total of forty-seven were found to have a serum cholesterol level exceeding 280 mg. per 100 ml. This group consisted of twenty-one men and twenty-six women, with an age range of 16 to 80 years, average 52.8 years. The serum cholesterol of these hypercholesteremic patients varied from 282 to 655 mg., average 338 mg. per 100 ml. For purposes of more detailed analysis, this group was subdivided into: (1) patients with disease commonly associated with hypercholesteremia; (2) patients with idiopathic hypercholesteremia whose families were not surveyed; (3) patients with idiopathic hypercholesteremia whose families were genetically normocholesteremic; and (4) patients who were members of hypercholesteremic families.

Fifteen of these forty-seven patients, five men and ten women, were suffering from diseases which are, in themselves, commonly associated with hypercholesteremia (Group I). Their ages ranged from 16 to 68 years, average 46.9 years. Their serum cholesterol levels varied from 282 to 655 mg., average 372 mg. per 100 ml. In these fifteen patients there were nine instances of intrahepatic or extrahepatic obstruction associated with icterus, three instances of nephrosis, and three endocrine disorders (Sheehan's syndrome, Cushing's syndrome, and uncontrolled diabetes).

Group II consisted of nine patients with elevated serum cholesterol levels not caused by their primary disease. The group included five men and four women. Their ages varied from 34 to 80 years, averaging 58.9 years. The total serum cholesterol ranged from 284 to 430 mg., average 326 mg. per 100 ml. In all of these patients the possibility of hereditary hypercholesteremia arose, but in each case further examination of the family was impossible. This was usually due to the fact that all the family members lived in widely separated areas throughout the country, and sometimes throughout the world. In two cases there was no living family member, and in one family the location of all members was unknown to the index patient. Occasionally an uncooperative individual was encountered who, without any stated reason, objected to being studied. This group emphasizes one of the greatest difficulties in a genetic study of this type, namely, the inability to obtain data on all family members.

Group III was composed of eleven index patients with idiopathic hypercholesteremia whose families were studied without finding adequate evidence for the presence of hereditary hypercholesteremia. A twelfth patient in whom

the suspicion of hereditary hypercholesteremia was aroused by the presence of corneal arcus, had a serum cholesterol of 260 mg. per 100 ml., but only two members of his immediate family proved to be hypercholesteremic (317 mg. and 333 mg. per 100 ml., respectively). There were then seven men and five women in Group III. Their ages varied from 38 to 66 years, average 54.1 years. The serum cholesterol ranged from 266 to 433 mg., average 317 mg. per 100 ml. In all, 41, or 58 per cent, of the seventy-one immediate surviving members of these twelve families were studied. The number of immediate family members studied varied from two to six per family with an average of 3.4.

Twelve patients had serum cholesterol levels in excess of 280 mg. per 100 ml., had no disease associated with hypercholesteremia, and had two or more relatives with hypercholesteremia (Group IV). The incidence of hereditary hypercholesteremia in the entire series of 200 families is then 6 per cent.

In establishing the criterion for the determination of hereditary hypercholesteremia, certain considerations had to be taken into account. If the random incidence of "idiopathic hypercholesteremia" in the general population were known, it could be compared to the results obtained in these families in order to determine whether hypercholesteremia was genetically transmitted. However, there is no known extensive study in which the random incidence of idiopathic hypercholesteremia has been estimated. Accordingly, this series of 200 index patients was used to ascertain the random incidence.

Forty-seven of the 200 index patients were hypercholesteremic. Fifteen patients had diseases commonly associated with elevated serum cholesterol levels and were excluded since they could not be considered to have idiopathic hypercholesteremia. Thirty-two of 185 patients, or 17.3 per cent, then, is computed to be the random incidence. Two instances of hypercholesteremia in immediate family members could occur with a random frequency of 3.0 per cent (17.3 per cent by 17.3 per cent). The possibility of hypercholesteremia being present, by chance, in three immediate members of a single family would be 0.5 per cent (17.3 per cent by 3.0 per cent).

A chance incidence of 0.5 per cent is infrequent enough to justify the genetic validity of the following criterion for the presence of hereditary hypercholesteremia; namely, the presence of this abnormality in three or more immediate family members. It must be emphasized, however, that more extensive observations may significantly alter the estimated random incidence of idiopathic hypercholesteremia; therefore the criterion for hereditary hypercholesteremia may have to be revised accordingly.

In this series, the observed incidence of 6 per cent has to be reduced to allow for random occurrence and thus the revised incidence of hereditary hypercholesteremia is 5.5 per cent. This incidence probably represents a minimum since some cases of hereditary hypercholesteremia remained undetected.

Table I presents the diagnoses, clinical data, and serum cholesterol levels of the index patients and the studied family members of Group IV. This group of twelve patients consisted of five men and seven women. Their ages varied from 37 to 80 years, averaging 55.5 years. The serum cholesterol levels ranged from 284 to 393 mg. per 100 ml. Of all ninety living family members in this group, 72,

TABLE I. PATIENTS WHO ARE MEMBERS OF HYPERCHOLESTEREMIC FAMILIES

PATIENT	SEX AGE COLOR	NATIVITY	RELIGION	DIAGNOSIS	PERTINENT CLINICAL AND LABORATORY FINDINGS	SERUM* CHOLESTEROL	EXAMINED FAMILY MEMBERS† SIBLINGS	CHILDREN	OTHERS‡	HYPERCHOLESTEREMIC, AVAILABLE AND SURVIVING FAMILY MEMBERS§
1. J. S.	M 50 W	Austria	J	Acute myocardial infarction, arteriosclerotic heart disease	ECG characteristics of acute myocardial infarction	327	S 54 286 S 52 205 B 45 345	Sn 20 212		3: 5: 5
2. D. G.	M 37 W	Argentina	J	Malignant hypertension, hypertensive encephalopathy	BP 192/126, ECG revealed myocardial damage	284	B 48 292 S 43 127 S 41 317			3: 4: 6
3. F. F.	M 58 W	Russia	J	Primary polycythemia, duodenal ulcer, diabetes mellitus, essential hypertension	BP 206/100, PZI 40 units daily	306	S 52 313 S 50 270	Sn 39 241 D 37 296 D 35 180	W 57 257	3: 6: 8
4. E. S.	M 48 W	Germany	G	Bronchiectasis	Corneal arcus, xanthelasma	303	B 52 360	D 22 328 Sn 20 185	W 43 280	3: 4: 11
5. M. W.	M 62 W	Germany	J	Acute myocardial infarction	BP 170/100, ECG characteristic of acute myocardial infarction	298	B 57 336	D 35 253 D 33 207 D 29 292 D 26 259		3: 6: 7
6. L. P.	F 48 W	U. S.	J	Kyphoscoliosis, kyphoscoliotic heart disease		329	S 51 323 B 47 225 S 44 281 S 38 237			3: 5: 5
7. A. D.	F 74 W	Russia	J	Acute myocardial infarction, arteriosclerotic and hypertensive cardiovascular disease	Corneal arcus, xanthelasma, ceased 24 hours after admission	340		Sn 57 321 D 54 376 Sn 52 331 Sn 46 318 Sn 44 312 D 40 293 Sn 39 349	GrD 27 312 GrD 23 331	8: 8: 8

TABLE I—CONT'D

PATIENT	SEX AGE COLOR	NATIVITY	RELIGION	DIAGNOSIS	PERTINENT CLINICAL AND LABORATORY FINDINGS	SERUM* CHOLESTEROL	EXAMINED FAMILY MEMBERS† SIBLINGS	CHILDREN	OTHERS‡	HYPERCHOLESTEREMIC, AVAILABLE AND SURVIVING FAMILY MEMBERS§

TABLE I—CONT'D

PATIENT	SEX AGE COLOR	NATIVITY	RELIGION	DIAGNOSIS	PERTINENT CLINICAL AND LABORATORY FINDINGS	SERUM* CHOLESTEROL	EXAMINED FAMILY MEMBERS† SIBLINGS	CHILDREN	OTHERS‡	HYPERCHOLESTEREMIC, AVAILABLE AND SURVIVING FAMILY MEMBERS§
8. B. L.	F 80 W	Russia	J	Arteriosclerotic and hypertensive cardiovascular disease, congestive heart failure	Corneal arcus, ECG revealed myocardial damage	301		D 53 286 D 47 295 D 45 247		3 : 4 : 5
9. S. G.	F 58 W	U. S.	J	Essential hypertension, obesity	BP 196/120	393	B 62 229 S 61 257 S 54 251 B 53 235 S 47 251 B 45 283 S 43 240 S 40 289	Sn 39 270 Sn 33 263 Sn 29 321		4 : 12 : 15
10. A. B.	F 63 W	Austria	J	Acute myocardial infarction, arteriosclerotic heart disease, congestive heart failure	ECG characteristic of acute myocardial infarction	340	B 67 419 B 58 350 B 53 317 B 46 336	D 38 226 D 32 281		6 : 7 : 8
11. D. N.	F 43 W	Russia	J	Rheumatic heart disease, inactive, cerebral embolus	Corneal arcus, BP 160/0, ECG revealed myocardial damage, died of cerebral embolus	286	S 56 322 B 54 369 S 52 349 B 50 270		Np 27 313	4 : 5 : 5
12. J. A.	F 46 W	U. S.	J	Paroxysmal auricular fibrillation, etiology undetermined		302	S 59 418 S 54 323 S 53 235 S 49 281 B 46 192			4 : 6 : 7

Abbreviations:

F—female; M—male; W—white; G—Gentile; J—Jewish; B—brother; S—sister; D—daughter; Sn—son; GrD—granddaughter; Np—nephew; Wf—wife; BP—blood pressure; ECG—electrocardiogram; PZI—protamine zinc insulin.

*Serum cholesterol expressed in mg. per 100 ml.

†Data of examined family members include relationship to index patient, age and total serum cholesterol level in that order.

‡The marital partner, whenever children of an index patient were examined, or occasionally relatives other than parents, siblings, or children of the index patient were examined. These persons, however, were excluded from consideration in determining the existence of hereditary hypercholesteremia.

§In this column the first figure indicates the number of hypercholesteremic immediate family members (parents, siblings, or children); the second, the number of examined family members; the third, the number of living immediate family members.

or 80 per cent, were studied. They included 47 siblings (66 per cent) and 25 children (34 per cent). Of these 72 members, 47, or 66 per cent, had hypercholesteremia. The number of immediate family members examined varied from four to twelve per family with an average of 6.0 members per family, whereas the number found to be hypercholesteremic ranged from three to eight with an average of 3.9 members per family. Two illustrative family trees are presented in Figs. 1 and 2.

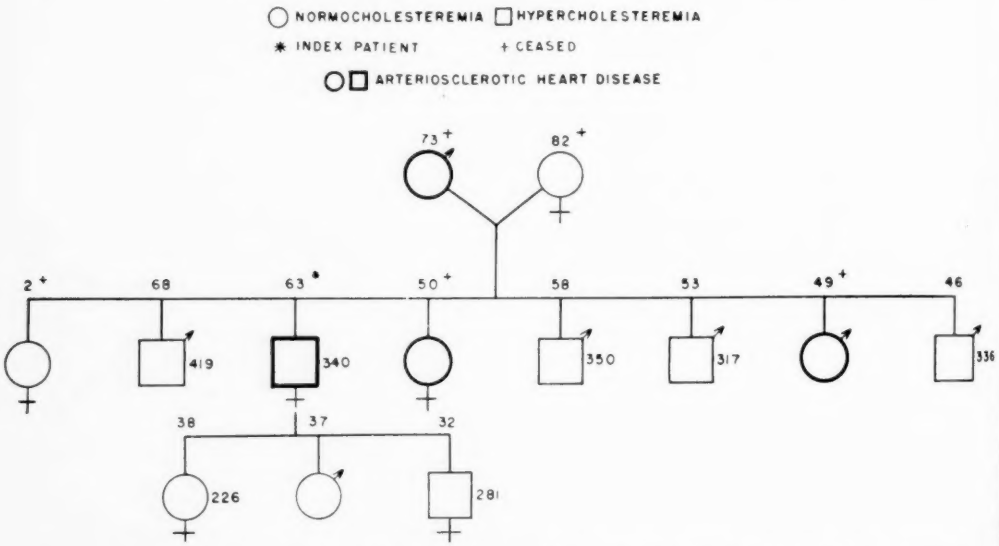

Fig. 1.—Patient 10, Table I. Six immediate surviving family members had hypercholesteremia. The index patient was hospitalized with a myocardial infarction. Her serum cholesterol level was 340 mg. per 100 ml. All four surviving siblings were hypercholesteremic. Two other siblings and the father, who are now dead, had arteriosclerotic heart disease. Of the index patient's three children, one proved to have an elevated serum cholesterol level.

All the patients in this group were white; not a single hypercholesteremic family was encountered among the nonwhite index patients who represented 10 per cent of the 200 patients examined. Only one (8 per cent) of the twelve hypercholesteremic families was Gentile, while the remaining 11 (92 per cent) were Jewish. In the entire group of 200 index patients, 45 per cent were non-Jewish and 55 per cent were Jewish, which is in agreement with the population ratio of this hospital. It is of interest that the basic disease was cardiovascular in nine of the twelve index patients, or 75 per cent. In the entire series of 200 index patients only 39 per cent fell into the cardiovascular-renal category.

COMMENT

This report, based on a study of 200 consecutive male and female admissions to a large general hospital, revealed an incidence of hereditary hypercholesteremia of 5.5 per cent. In reviewing the results of this study, a few general criticisms should be considered.

The ideal condition for a survey study of this nature is a completely unselected group of families representative of the general population. This require-

ment was not fulfilled in our series, since the "index" family member had an illness, usually of a serious nature, which required hospitalization. The serum cholesterol levels of these index patients were elevated or depressed in many instances, by their disease. Consequently in patients with conditions which are commonly associated with hypercholesteremia, it was impossible to determine whether a normal or elevated serum cholesterol level was present prior to the onset of the disease. Pyrexia, especially when high or of long duration, and diseases producing cachexia are known to lower the total serum cholesterol level. Under these conditions, idiopathic hypercholesteremia might have been masked since it was often impossible to perform serum cholesterol determinations after the patient's recovery.

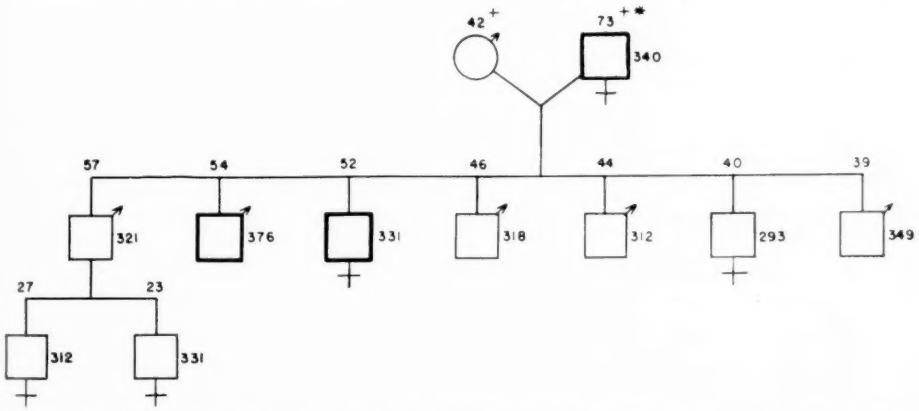

Fig. 2.—Patient 7, Table I. Eight immediate family members (omitting two grandchildren of the index patient from consideration) had elevated serum cholesterol levels. The index patient died in the hospital. Post-mortem examination revealed myocardial infarction and severe systemic atherosclerosis. The serum cholesterol level of the index patient was 340 mg. per 100 ml. All seven of her children were examined and were found to be hypercholesteremic. Two of these children had arteriosclerotic heart disease and two others had mild diabetes mellitus. Two grandchildren, ages 27 and 23 years, exhibited hypercholesteremia.

It must be kept in mind that the population of this hospital is not truly representative of New York City, the site of study. In a wider, more important sense, the population of the hospital is not a representation of the population of the United States in that racial origins and environmental factors (climate, diet, occupation, etc.) differ significantly from those of other areas. It would be of great value, therefore, to assay population groups in other parts of the country in order to obtain a more accurate estimate of the incidence of hereditary hypercholesteremia.

These observations revealed two possible factors concerning hereditary hypercholesteremia. There was no proved instance of hereditary hypercholesteremia in the nonwhite families. Only one nonwhite patient had idiopathic hypercholesteremia but a study of his family was not practicable since all surviving family members resided in Puerto Rico.

It is also of interest to note the high incidence of hereditary hypercholesteremia among the patients of Jewish descent. In 104 Jewish families there were eleven instances of proved hereditary hypercholesteremia (10.6 per cent),

whereas in 96 non-Jewish families, this abnormality was encountered in only one instance (1.0 per cent). Although these differences may be coincidental because of the limited number of observed families, it should be recalled that other inborn errors of lipid metabolism, such as Niemann-Pick's disease, Gaucher's disease and Tay-Sach's disease are frequently encountered among the Jewish people.[7b] In this connection, the not infrequent occurrence of consanguineous marriages and "assortative matings" in the small Jewish communities of the European continent might have been factors contributing to the high incidence of inherited errors of lipid metabolism in the Jewish segment of the population.

This survey represents the first attempt to estimate the occurrence of hereditary hypercholesteremia in the population. It may be recalled that a survey of the population of a single community, Oxford, Massachusetts, for the occurrence of diabetes mellitus[9] revealed a higher than expected incidence of this metabolic disorder. The incidence of hereditary hypercholesteremia, as judged by this survey, also appears to be higher than expected.

In view of the possible relationship between hypercholesteremia and atherosclerosis, which is more frequently observed in a progressively aging population, these genetic studies assume an added significance.

SUMMARY

1. A study of the families of 200 consecutive unselected admissions, 100 men and 100 women, to the medical wards of a large general hospital revealed an incidence of hereditary hypercholesteremia of 5.5 per cent.

2. Hereditary hypercholesteremia was judged to be present when three or more immediate family members were found to have a total serum cholesterol level in excess of 280 mg. per 100 ml. as determined by the Sperry-Schoenheimer method.

3. Certain considerations which may have elevated or reduced the estimated incidence of this hereditary disorder were discussed.

4. The high incidence of hereditary hypercholesteremia among Jewish families, and the absence of this abnormality among the nonwhite segment in this limited series, were noted.

5. This survey is being extended to include unselected families from the population at large.

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VARIATIONS IN ANTI-HUMAN GLOBULIN TITRATION RESULTS

Method for Detecting Rh_0 Antibodies in Extremely Low Titer

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THE anti-human globulin test (Coombs' test)[1] is one of various methods for detecting univalent Rh_0 antibodies. Although this method has proved of great value, there are conflicting opinions as to its sensitivity compared with that of other tests. Morton and Pickles[2] state that the trypsinated cell method[3] is more sensitive than the indirect antiglobulin method. Rosenfield[4] finds that titers by the two methods are by and large the same, although at times one method or the other gives higher titers for a given serum, and such results are reproducible. In our own hands, in most instances, higher titers have been observed with the indirect antiglobulin method than with the trypsinated cell method. Furthermore, we have found that the trypsinated cell indirect antiglobulin method,[5] which is really a combination of the two methods, usually gives titer values equal to or slightly higher than those obtained by the indirect antiglobulin method. The purpose of this communication is to demonstrate the relationship of the titer of the anti-human globulin serum employed to the results obtained.

METHODS AND MATERIALS

The anti-human globulin test makes use of immune rabbit serum which will agglutinate human erythrocytes, "sensitized" or "coated" with human globulin such as Rh_0-positive cells which have been sensitized with univalent Rh_0 antibodies. If the test is carried out with Rh_0-positive cells which have combined in vivo with antibodies specific for this factor, it is called the direct antiglobulin test. If sensitization of red cells is made to take place in vitro, the test is called the indirect antiglobulin test. In this investigation the latter procedure was studied.

Six anti-human globulin serums from six different sources were selected. With each serum a double set of cross-titrations was carried out. In one set sensitized untreated red cells were used and in the other, cells which were trypsinated prior to sensitization. In each individual double set of cross-titrations, the same test cells were used. Blood for the tests was always freshly drawn. In two of the sets, the test cell belonged to group O Rh_1rh, in three they were group O Rh_1Rh_1, and in one group O Rh_2rh. For each titration group O rh cells exposed to the same anti-Rh_0 serum were used as controls as well as group A and group B cells without exposure to the anti-Rh_0 serum. Three antiglobulin serums were of commercial origin and therefore had been absorbed and diluted ready for use before distribution. Three other serums were prepared from immunized rabbit's clotted blood, diluted 1:2 and then absorbed with thoroughly washed (five times with saline) pooled fresh group O, A, B, and AB human red blood cells (all Rh-positive) in order to remove anti-human hemagglutinins present in rabbit serum. In each case several absorptions were necessary and the serum was not considered absorbed until it was no longer able to clump the absorbing cells.

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Preparation of Test Cells.—The cells used for the titrations were washed four times in normal saline solution, packed and then divided into two parts. One part was resuspended in normal saline to make a 2 per cent suspension of cells and labelled "untreated cells." With nine parts of the remainder of washed cells* was mixed one part of 1 per cent trypsin (Difco) in standard buffer solution† and placed in a water bath at 37° C. for one hour, resuspending the cells every fifteen minutes, after which they were washed once in normal saline to remove trypsin. The saline was then decanted and the cell sediment made up as a 2 per cent saline suspension and the tube labelled "trypsinated cells."

Preparation of Dilutions of Univalent Rh_o Serum.—To sensitize both the untreated and trypsinated cells, univalent anti-Rh_o serum was used. The titer values of this serum were 80 units by the albumin-plasma method, 160 units by the trypsinated cell method, and 6 units by the blocking method. The serum did not agglutinate untreated saline suspended cells. This single serum was used throughout the entire experiment in order to eliminate this source of variation. The serum was diluted 1:10 with normal saline solution and then progressively doubled saline dilutions were made ranging up to 1:5120. The calculated titer of each successive serum dilution was thus half that of the preceding one and ranged from 8 units to $\frac{1}{64}$ of a unit in terms of the albumin-plasma titer of the undiluted serum. Each of these dilutions was labelled with the calculated titer, and then used separately to sensitize group O Rh-positive test cells, both untreated and trypsinated.

Cross-titration Using Untreated Cells.—In each of ten test tubes were placed ten drops of 2 per cent saline suspended "untreated cells." To the first tube was added ten drops of serum dilution, the titer of which was 8 units; to the second tube ten drops of the 4-unit dilution, and so on until the tenth dilution ($\frac{1}{64}$ of a unit) had been added. These tubes were placed in a water bath at 37° C. for one hour after which the cells in each test tube were washed four times in saline solution and then resuspended to make a 2 per cent suspension of sensitized untreated cells. One hundred small test tubes (8 mm. in diameter) were arranged in racks in ten rows of ten tubes each. Into the first tube of each row was placed one drop of 2 per cent saline suspended untreated cells, sensitized with the most concentrated dilution of anti-Rh_o serum. Into the second tube of each row was placed one drop of 2 per cent saline suspended untreated cells sensitized with the next higher dilution of anti-Rh_o serum. This process was continued for each succeeding tube of every row until all ten sets of cells sensitized with anti-Rh_o serum of decreasing antibody titer had been added. The hundred tubes were then centrifuged and the saline removed. To each tube of the first row, one drop of 1:2 dilution of antiglobulin serum was added and then mixed; to each tube of the second row, one drop of 1:4 dilution of antiglobulin was mixed. This was continued until all ten dilutions of antiglobulin serum had been added to the corresponding rows. The one hundred tubes were centrifuged lightly (500 to 1,000 r.p.m.) for one minute and the sediment gently resuspended and examined *in the tube* for clumping, using the lowest power (scanning lens) of the microscope. Some workers carry out the test on a slide or "tile," but we find this less sensitive than the test tube method. The titer of the anti-Rh_o serum was taken equal to the reciprocal of the highest dilution of serum used to sensitize cells which gave a distinct (1+) reaction with the antiglobulin serum dilution used in each instance.

Cross-titration Using Trypsinated Cells.—For this it was necessary to sensitize cells in *individual* tubes rather than en masse as just described, because univalent Rh_o antibodies clump saline suspended trypsinated Rh_o-positive cells. For this reason, into each of the one hundred small tubes (8 mm. in diameter), arranged as already described with ten tubes in each of ten rows, was placed one drop of 2 per cent saline suspended trypsinated group O Rh-positive cells. To the first tube of each row was added one drop of anti-Rh_o serum dilution of the highest titer. To the second tube of each row was added one drop of the next higher dilution of this serum, etc., until all ten anti-Rh_o serum dilutions of decreasing antibody titers were used. The one hundred tubes were placed in a water bath at 37° C. for one hour after which the sediment in each tube was examined for clumping using the lowest power of the microscope (scanning lens). Since the titer of the anti-Rh_o serum used, prior to dilution was

*Washed four times with normal saline to remove natural antitrypsin.
†M/15 Phosphate Buffer Solution pH 7.41.

160 units, by the trypsinated cell method, the cells were clumped in the first five tubes of each row because the trypsinated cells in each of these tubes had been exposed to univalent anti-Rh_0 serum in dilutions varying from 1:10 to 1:160. On the unclumped cells in all remaining tubes an antiglobulin test was done as previously described, and the cells in each tube were then examined for clumping and read as in the previous test.

RESULTS

Tables I, II, and III show the results of three double sets of cross-titrations using three different commercial antiglobulin serums each obtained from a different source. Each double set of cross-titrations is actually a series of titrations of the same univalent anti-Rh_0 serum, one by the standard indirect antiglobulin and the other by the trypsinated cell indirect antiglobulin method using progressively doubled dilutions of antiglobulin serum. Commercial antiglobulin serums must meet certain minimum standards set by the National Institute of Health. This governmental agency requires that "anti-human serum shall give a 1+ agglutination of red cells exposed to the greatest dilution of the anti-Rh_0 (anti-D) serum which will react with those red cells in albumin or comparable diluent," and that "the finished product when diluted at least 1:4 with saline shall give no less titer value for the anti-Rh_0 (anti-D) antibody

TABLE I. RESULTS OF TWO CROSS-TITRATIONS OF UNIVALENT ANTI-RH_0 SERUM WITH A COMMERCIAL ANTI-HUMAN GLOBULIN SERUM (No. 1)

	SALINE DILUTIONS OF ANTI-HUMAN GLOBULIN SERUM	SALINE DILUTIONS OF UNIVALENT ANTI-RH_0 SERUM USED TO SENSITIZE THE TEST CELLS										TITER OF RH_0 SERUM
		1:10 (8)*	1:20 (4)	1:40 (2)	1:80 (1)	1:160 (½)	1:320 (¼)	1:640 (⅛)	1:1280 (1/16)	1:2560 (1/32)	1:5120 (1/64)	
Untreated Cells	Undiluted	++s	++	+±	+s	+s	±	–	–	–	–	200
	1:2	++	++	+±	+s	+	–	–	–	–	–	160
	1:4	++	++w	++w	+±	–	–	–	–	–	–	120
	1:8	++	++	++w	+s	–	–	–	–	–	–	100
	1:16	++	+±	+s	–	–	–	–	–	–	–	50
	1:32	++	+	–	–	–	–	–	–	–	–	20
	1:64	+s	±	–	–	–	–	–	–	–	–	10
	1:128	tr	–	–	–	–	–	–	–	–	–	0
	1:256	–	–	–	–	–	–	–	–	–	–	0
	1:512	–	–	–	–	–	–	–	–	–	–	0
Trypsinated Cells	Undiluted	**	**	**	**	**	+w	–	–	–	–	250
	1:2	**	**	**	**	**	+w	–	–	–	–	250
	1:4	**	**	**	**	**	±	–	–	–	–	***
	1:8	**	**	**	**	**	–	–	–	–	–	***
	1:16	**	**	**	**	**	–	–	–	–	–	***
	1:32	**	**	**	**	**	–	–	–	–	–	***
	1:64	**	**	**	**	**	–	–	–	–	–	***
	1:128	**	**	**	**	**	–	–	–	–	–	***
	1:256	**	**	**	**	**	–	–	–	–	–	***
	1:512	**	**	**	**	**	–	–	–	–	–	***

The reactions are rated by the number of plus signs. A three-plus reaction represents the maximum reactions possible, namely, one solid clump. The symbols s, w, and tr stand for strong, weak, and trace, respectively.

*The titer values of the undiluted anti-Rh_0 serum were 80 units by the albumin-plasma, 160 units by the trypsinated cell, 6 units by the blocking and negative by the saline agglutination methods. The figures in the parentheses are the calculated titers (in units or fractions thereof) of each dilution of the univalent anti-Rh_0 serum used to coat the test cells based on the titer of the undiluted serum (80 units) as determined by the albumin-plasma method.

**The trypsinated cells in these tubes were clumped by the anti-Rh_0 serum and therefore no antiglobulin test was carried out.

***Since no coating was detected by the antiglobulin serum in these weak concentrations, no titer values could be determined.

TABLE II. RESULTS OF TWO CROSS-TITRATIONS OF UNIVALENT ANTI-Rh_o SERUM WITH A COMMERCIAL ANTI-HUMAN GLOBULIN SERUM (NO. 2)

	SALINE DILUTIONS OF ANTI-HUMAN GLOBULIN SERUM	SALINE DILUTIONS OF UNIVALENT ANTI-RH_o SERUM USED TO SENSITIZE THE TEST CELLS										TITER OF RH_o SERUM
		1:10 (8)*	1:20 (4)	1:40 (2)	1:80 (1)	1:160 (½)	1:320 (¼)	1:640 (⅛)	1:1280 (1/16)	1:2560 (1/32)	1:5120 (1/64)	
Untreated Cells	Undiluted	+++	++s	++	++w	+s	tr	–	–	–	–	200
	1:2	++s	++	++w	++w	+s	–	–	–	–	–	200
	1:4	++	++	++w	+±	+	–	–	–	–	–	160
	1:8	++	+±	+±	+±	–	–	–	–	–	–	120
	1:16	++w	+±	+	–	–	–	–	–	–	–	40
	1:32	+	tr	–	–	–	–	–	–	–	–	15
	1:64	–	–	–	–	–	–	–	–	–	–	0
	1:128	–	–	–	–	–	–	–	–	–	–	0
	1:256	–	–	–	–	–	–	–	–	–	–	0
	1:512	–	–	–	–	–	–	–	–	–	–	0
Trypsinated Cells	Undiluted	**	**	**	**	**	+	–	–	–	–	320
	1:2	**	**	**	**	**	+	–	–	–	–	320
	1:4	**	**	**	**	**	+	–	–	–	–	320
	1:8	**	**	**	**	**	tr	–	–	–	–	***
	1:16	**	**	**	**	**	–	–	–	–	–	***
	1:32	**	**	**	**	**	–	–	–	–	–	***
	1:64	**	**	**	**	**	–	–	–	–	–	***
	1:128	**	**	**	**	**	–	–	–	–	–	***
	1:256	**	**	**	**	**	–	–	–	–	–	***
	1:512	**	**	**	**	**	–	–	–	–	–	***

The reactions are rated by the number of plus signs. A three-plus reaction represents the maximum reactions possible, namely, one solid clump. The symbols s, w, and tr stand for strong, weak, and trace, respectively.

*The titer values of the undiluted anti-Rh_o serum were 80 units by the albumin-plasma, 160 units by the trypsinated cell, 6 units by the blocking and negative by the saline agglutination methods. The figures in the parentheses are the calculated titers (in units or fractions thereof) of each dilution of the univalent anti-Rh_o serum used to coat the test cells based on the titer of the undiluted serum (80 units) as determined by the albumin-plasma method.

**The trypsinated cells in these tubes were clumped by the anti-Rh_o serum and therefore no antiglobulin test was carried out.

***Since no coating was detected by the antiglobulin serum in these weak concentrations, no titer values could be determined.

than the undiluted anti-human serum." Antiglobulin serums just meeting these minimum requirements should yield titer values equal to or slightly higher than those obtained by the albumin-plasma method and approximately the same as for the trypsinated cell method as done by us. The first two examples of antiglobulin serum tested (Tables I and II) were approximately of equal strength and the third example (Table III) gave a higher titer value. All three antiglobulin serums exceeded the minimum requirements of the N. I. H. These serums had been released more potent than required, probably to provide against any possible subsequent deterioration.

The experiments were repeated using three highly concentrated antiglobulin serums. The highest concentration tested was 1:2, because the serums had already been diluted 1:2 preliminary to the process of absorbing anti-human hemagglutinins present in rabbit serum. Starting with this dilution, double sets of cross-titrations were carried out in the same fashion as with the commercial serums. Tables IV, V, and VI give the results of tests using these three different antiglobulin serums for titrations of the same anti-Rh_o serum which had been used in the previous experiments. When these antiglobulin serums were used in the most concentrated dilution, the titers for untreated cells were 1,280,

TABLE III. RESULTS OF TWO CROSS-TITRATIONS OF UNIVALENT ANTI-RH$_0$ SERUM WITH A COMMERCIAL ANTI-HUMAN GLOBULIN SERUM (NO. 3)

	SALINE DILUTIONS OF ANTI-HUMAN GLOBULIN SERUM	SALINE DILUTIONS OF UNIVALENT ANTI-RH$_0$ SERUM USED TO SENSITIZE THE TEST CELLS										TITER OF RH$_0$ SERUM
		1:10 (8)*	1:20 (4)	1:40 (2)	1:80 (1)	1:160 (½)	1:320 (¼)	1:640 (⅛)	1:1280 (1/16)	1:2560 (1/32)	1:5120 (1/64)	
Untreated Cells	Undiluted	++s	++s	++s	++s	++	+s	–	–	–	–	400
	1:2	++s	++s	++	++	++w	+s	–	–	–	–	400
	1:4	++s	++s	++	++	+s	+	–	–	–	–	320
	1:8	++s	++	++	++	+s	+w	–	–	–	–	240
	1:16	++s	++	++w	+s	+	+w	–	–	–	–	240
	1:32	++	++w	++w	+s	+w	–	–	–	–	–	120
	1:64	+s	+	–	–	–	–	–	–	–	–	20
	1:128	–	–	–	–	–	–	–	–	–	–	0
	1:256	–	–	–	–	–	–	–	–	–	–	0
	1:512	–	–	–	–	–	–	–	–	–	–	0
Trypsinated Cells	Undiluted	**	**	**	**	**	+s	+s	–	–	–	800
	1:2	**	**	**	**	**	++w	–	–	–	–	400
	1:4	**	**	**	**	**	++w	–	–	–	–	400
	1:8	**	**	**	**	**	+s	–	–	–	–	400
	1:16	**	**	**	**	**	++w	–	–	–	–	400
	1:32	**	**	**	**	**	+w	–	–	–	–	240
	1:64	**	**	**	**	**	–	–	–	–	–	***
	1:128	**	**	**	**	**	–	–	–	–	–	***
	1:256	**	**	**	**	**	–	–	–	–	–	***
	1:512	**	**	**	**	**	–	–	–	–	–	***

The reactions are rated by the number of plus signs. A three-plus reaction represents the maximum reactions possible, namely, one solid clump. The symbols s, w, and tr stand for strong, weak, and trace, respectively.

*The titer values of the undiluted anti-Rh$_0$ serum were 80 units by the albumin-plasma, 160 units by the trypsinated cell, 6 units by the blocking and negative by the saline agglutination methods. The figures in the parentheses are the calculated titers (in units or fractions thereof) of each dilution of the univalent anti-Rh$_0$ serum used to coat the test cells based on the titer of the undiluted serum (80 units) as determined by the albumin-plasma method.

**The trypsinated cells in these tubes were clumped by the anti-Rh$_0$ serum and therefore no antiglobulin test was carried out.

***Since no coating was detected by the antiglobulin serum in these weak concentrations, no titer values could be determined.

400, and 640 units, respectively, and for trypsin-treated cells 2,560, 1,280, and 640 units, respectively, in contrast to a titer of 160 units obtained by the trypsinated cell method. In order to indicate the combinations which yielded positive reactions by the standard indirect antiglobulin and the trypsinated cell indirect antiglobulin methods, though negative by the trypsinated cell method, the reactions in question are outlined by a heavy black line (see tables). It can be seen from the tables (see Tables I through VI) that the concentration of antiglobulin serum plays an important role in determining the extent to which titers obtained with such serum surpass those obtained by the trypsinated cell method. The tables also show that a slightly higher titer is obtained by the trypsinated cell antiglobulin method, than by the standard antiglobulin method.

Table VII is a summary comparing the titers obtained with the six examples of antiglobulin serums, each at two different strengths. When the three commercial antiglobulin serums were used as supplied, titers by the standard antiglobulin method were higher than by the albumin-plasma method and approximately the same or higher than the trypsinated cell method. In one instance the titer by the trypsinated cell antiglobulin method was higher by one tube than by the standard antiglobulin method; in the other two they were approximately

TABLE IV. RESULTS OF TWO CROSS-TITRATIONS OF UNIVALENT ANTI-Rh_o SERUM WITH A COMMERCIAL ANTI-HUMAN GLOBULIN SERUM (NO. 4)

	SALINE DILUTIONS OF ANTI-HUMAN GLOBULIN SERUM	SALINE DILUTIONS OF UNIVALENT ANTI-Rh_o SERUM USED TO SENSITIZE THE TEST CELLS										TITER OF Rh_o SERUM
		1:10 (8)*	1:20 (4)	1:40 (2)	1:80 (1)	1:160 (1/2)	1:320 (1/4)	1:640 (1/8)	1:1280 (1/16)	1:2560 (1/32)	1:5120 (1/64)	
Untreated Cells	1:2	++±	++s	+++	++s	++w	+±	+s	±	−	−	1280
	1:4	++s	+++	++s	++s	++w	+s	+	−	−	−	640
	1:8	++s	++s	++s	++	++w	+s	+	−	−	−	400
	1:16	++s	++s	++	++	+±	+s	tr	−	−	−	400
	1:32	++s	++s	++	++	+±	+s	−	−	−	−	320
	1:64	++s	++s	++	++w	+s	+w	−	−	−	−	200
	1:128	++	++	++w	+±	+	−	−	−	−	−	160
	1:256	++w	++w	+±	+s	−	−	−	−	−	−	100
	1:512	+±	+±	+	+w	−	−	−	−	−	−	50
	1:1024	+s	+	tr	−	−	−	−	−	−	−	20
Trypsinated Cells	1:2	**	**	**	**	**	+++	++s	++s	+	−	2560
	1:4	**	**	**	**	**	++s	++	++w	+	−	2560
	1:8	**	**	**	**	**	++	++w	+±	−	−	1900
	1:16	**	**	**	**	**	+±	+s	+w	−	−	800
	1:32	**	**	**	**	**	+s	−	−	−	−	400
	1:64	**	**	**	**	**	−	−	−	−	−	***
	1:128	**	**	**	**	**	−	−	−	−	−	***
	1:256	**	**	**	**	**	−	−	−	−	−	***
	1:512	**	**	**	**	**	−	−	−	−	−	***
	1:1024	**	**	**	**	**	−	−	−	−	−	***

The reactions are rated by the number of plus signs. A three-plus reaction represents the maximum reactions possible, namely, one solid clump. The symbols s, w, and tr stand for strong, weak, and trace, respectively.

*The titer values of the undiluted anti-Rh_o serum were 80 units by the albumin-plasma, 160 units by the trypsinated cell, 6 units by the blocking and negative by the saline agglutination methods. The figures in the parentheses are the calculated titers (in units or fractions thereof) of each dilution of the univalent anti-Rh_o serum used to coat the test cells based on the titer of the undiluted serum (80 units) as determined by the albumin-plasma method.

**The trypsinated cells in these tubes were clumped by the anti-Rh_o serum and therefore no antiglobulin test was carried out.

***Since no coating was detected by the antiglobulin serum in these weak concentrations, no titer values could be determined.

the same. When the commercial antiglobulin serums were further diluted, the titers obtained were quite low and such diluted antiglobulin serums were unable to detect coating of cells which previously had been trypsinated. This, of course, demonstrates the value of the control methods exercised by the N. I. H. These results show that the trypsinated cell method yields titers higher than the standard antiglobulin method if *weak* antiglobulin serum is used, and that with such weak antiglobulin serum the trypsinated cell antiglobulin method becomes insensitive. Table VII also shows the different titer values obtained with three examples of noncommercial antiglobulin serum used at two different strengths. Obviously, titers obtained by the antiglobulin method (using untreated or trypsinated cells) can, within limits, be changed depending upon the strength of antibodies in the original undiluted immune antiglobulin rabbit serum and upon the degree to which this serum is diluted. There are three variables for the antiglobulin method; cells, Rh antibodies and antiglobulin serum. The antiglobulin method is a valuable one and it would seem reasonable, therefore, if we are to obtain the maximum potentialities of the test, more highly concentrated antiglobulin be used than is presently supplied commercially, and that the test be carried out in *test tubes* rather than on a tile. Concentrated anti-

TABLE V. RESULTS OF TWO CROSS-TITRATIONS OF UNIVALENT ANTI-Rh_o SERUM WITH A COMMERCIAL ANTI-HUMAN GLOBULIN SERUM (No. 5)

	SALINE DILUTIONS OF ANTI-HUMAN GLOBULIN SERUM	SALINE DILUTIONS OF UNIVALENT ANTI-Rh_o SERUM USED TO SENSITIZE THE TEST CELLS										TITER OF Rh_o SERUM
		1:10 (8)*	1:20 (4)	1:40 (2)	1:80 (1)	1:160 (1/2)	1:320 (1/4)	1:640 (1/8)	1:1280 (1/16)	1:2560 (1/32)	1:5120 (1/64)	
Untreated Cells	1:2	++s	++s	++	++w	++w	+s	–	–	–	–	400
	1:4	++s	++	++	+±	+s	+	–	–	–	–	320
	1:8	++s	++	++	+±	+	+	–	–	–	–	320
	1:16	++	++	++w	+±	+	–	–	–	–	–	160
	1:32	++	++w	+±	+	–	–	–	–	–	–	80
	1:64	++w	+±	+	–	–	–	–	–	–	–	40
	1:128	+s	+	tr	–	–	–	–	–	–	–	20
	1:256	+w	–	–	–	–	–	–	–	–	–	10
	1:512	–	–	–	–	–	–	–	–	–	–	0
	1:1024	–	–	–	–	–	–	–	–	–	–	0
Trypsinated Cells	1:2	**	**	**	**	**	++w	++w	+	–	–	1280
	1:4	**	**	**	**	**	+±	+s	±	–	–	800
	1:8	**	**	**	**	**	+±	+s	–	–	–	800
	1:16	**	**	**	**	**	tr	–	–	–	–	***
	1:32	**	**	**	**	**	–	–	–	–	–	***
	1:64	**	**	**	**	**	–	–	–	–	–	***
	1:128	**	**	**	**	**	–	–	–	–	–	***
	1:256	**	**	**	**	**	–	–	–	–	–	***
	1:512	**	**	**	**	**	–	–	–	–	–	***
	1:1024	**	**	**	**	**	–	–	–	–	–	***

The reactions are rated by the number of plus signs. A three-plus reaction represents the maximum reactions possible, namely, one solid clump. The symbols s, w, and tr stand for strong, weak, and trace, respectively.

*The titer values of the undiluted anti-Rh_o serum were 80 units by the albumin-plasma, 160 units by the trypsinated cell, 6 units by the blocking and negative by the saline agglutination methods. The figures in the parentheses are the calculated titers (in units or fractions thereof) of each dilution of the univalent anti-Rh_o serum used to coat the test cells based on the titer of the undiluted serum (80 units) as determined by the albumin-plasma method.

**The trypsinated cells in these tubes were clumped by the anti-Rh_o serum and therefore no antiglobulin test was carried out.

***Since no coating was detected by the antiglobulin serum in these weak concentrations, no titer values could be determined.

globulin serum can be supplied within the framework of the N. I. H. regulations which are minimum standards. Beyond the end-point of a trypsinated cell titration of most, although not all anti-Rh_o serums, for one or more dilutions, cells may be coated but not clumped. Coating of these cells can be demonstrated by the antiglobulin test, provided the antiglobulin serum used is sufficiently potent. It is known that some examples of high-titered antiglobulin serum give a prozone effect,[6] when tested against weakly sensitized cells and this must be taken into account when appraising the value of each individual antiglobulin serum. Perhaps it would be better that serums which show a prozone be avoided.

If antiglobulin serums with regularity gave results of no higher sensitivity than other methods, such as the conglutination or trypsinated cell methods, they would add no information but merely duplicate effort. But, as just shown, antiglobulin serums can be prepared so that they will detect Rh_o antibodies of such low titer that other methods fail. Therefore, in our laboratory, instead of being satisfied with commercial antiglobulin serums, we use much more potent serums. This conforms with the opinion of Roberts[7] who states "the most important risk is the use of an antiserum which is too weak." He recommends that strong serums be prepared with alum-precipitated antigens as described by Coombs and Mourant.[8]

TABLE VI. RESULTS OF TWO CROSS-TITRATIONS OF UNIVALENT ANTI-Rh_o SERUM WITH A COMMERCIAL ANTI-HUMAN GLOBULIN SERUM (No. 6)

	SALINE DILUTIONS OF ANTI-HUMAN GLOBULIN SERUM	SALINE DILUTIONS OF UNIVALENT ANTI-Rh_o SERUM USED TO SENSITIZE THE TEST CELLS										TITER OF Rh_o SERUM
		1:10 (8)*	1:20 (4)	1:40 (2)	1:80 (1)	1:160 (1/2)	1:320 (1/4)	1:640 (1/8)	1:1280 (1/16)	1:2560 (1/32)	1:5120 (1/64)	
Untreated Cells	1:2	+++	++s	++s	++	++w	±	+w	–	–	–	640
	1:4	+++	++s	++s	++	±	+s	–	–	–		400
	1:8	+++	++s	++	++w	+s	+w	–	–	–	–	300
	1:16	++±	++s	++	++w	+s	±	–	–	–	–	200
	1:32	++s	++s	++w	++w	±	–	–	–	–	–	240
	1:64	++	++w	++w	±	+w	–	–	–	–	–	150
	1:128	++w	++w	+s	±	–	–	–	–	–	–	60
	1:256	±	±	+	–	-	–	–	–	–	–	40
	1:512	+w	–	–	–	–	–	–	–	–	–	10
	1:1024	–	–	–	–	–	–	–	–	–	–	0
Trypsinated Cells	1:2	**	**	**	**	**	++w	+	±	–	–	640
	1:4	**	**	**	**	**	++w	+	–	–	–	640
	1:8	**	**	**	**	**	±	+	–	–	–	640
	1:16	**	**	**	**	**	++w	+w	–	–	–	560
	1:32	**	**	**	**	**	+	tr	–	–	–	320
	1:64	**	**	**	**	**	±	–	–	–	–	320
	1:128	**	**	**	**	**	–	–	–	–	–	***
	1:256	**	**	**	**	**	–	–	–	–	–	***
	1:512	**	**	**	**	**	–	–	–	–	–	***
	1:1024	**	**	**	**	**	–	–	–	–	–	***

The reactions are rated by the number of plus signs. A three-plus reaction represents the maximum reactions possible, namely, one solid clump. The symbols s, w, and tr stand for strong, weak, and trace, respectively.

*The titer values of the undiluted anti-Rh_o serum were 80 units by the albumin-plasma, 160 units by the trypsinated cell, 6 units by the blocking and negative by the saline agglutination methods. The figures in the parentheses are the calculated titers (in units or fractions thereof) of each dilution of the univalent anti-Rh_o serum used to coat the test cells based on the titer of the undiluted serum (80 units) as determined by the albumin-plasma method.

**The trypsinated cells in these tubes were clumped by the anti-Rh_o serum and therefore no antiglobulin test was carried out.

***Since no coating was detected by the antiglobulin serum in these weak concentrations, no titer values could be determined.

Trypsin-treatment of cells[9] is beyond doubt a useful procedure. It permits saline suspended cells to be clumped by specific univalent antibodies; it eliminates the prozone effect of some serums; it gives sharp end-points; it may detect univalent antibodies present in serum in such low titer that the albumin-plasma method fails; and when used with the antiglobulin method it may detect Rh_o antibodies in a titer beyond the range of other methods. Trypsin seems to have at least two effects on erythrocytes. It makes them sticky, seemingly substituting for protein media or high molecular media necessary for the conglutination test, and also acts on agglutinogens, although this latter action varies with the factor. It increases the avidity of certain agglutinogens for their specific antibodies, as the Rh factor; it has a destructive effect on other agglutinogens, namely Duffy,[10] M, and N;[11] it has no effect on still others such as Kell and Kidd.[11]

This raises the question of the significance of antibodies present in extremely low titers. From a scientific point of view, quite apart from its clinical importance, it is desirable to obtain all possible information and for that reason it is desirable to know whether or not an individual is sensitized, irrespective of the degree. If a particular method yields this information and others do not, it should be used. From a clinical point of view, as far as erythroblastosis is

TABLE VII. TITERS (IN UNITS) OF A UNIVALENT ANTI-Rh_0 SERUM USING SIX EXAMPLES OF ANTI-HUMAN GLOBULIN SERUM (SUMMARY OF TABLES I THROUGH VI)

METHODS OF TITRATION	COMMERCIAL ANTIGLOBULIN SERUMS						NONCOMMERCIAL ANTIGLOBULIN SERUMS					
	AS SUPPLIED, SLIGHTLY STRONGER THAN N. I. H. REQUIREMENTS			DILUTED* SLIGHTLY WEAKER THAN N. I. H. REQUIREMENTS			MAXIMUM CONCENTRATION (DILUTED 1:2)			DILUTED TO N. I. H. REQUIREMENTS		
	SERUM #1	SERUM #2	SERUM #3	SERUM #1	SERUM #2	SERUM #3	SERUM #4	SERUM #5	SERUM #6	SERUM #4	SERUM #5	SERUM #6
Indirect antiglobulin (untreated cells)	200	200	400	50	50	20	1,280	400	640	200	320	200
Trypsinated cell indirect antiglobulin	250	320	800	**	**	**	2,560	1,280	640	**	800	560

The titer was taken as the reciprocal of the highest dilution of anti-Rh_0 serum used to coat those test cells which gave a distinct reaction (1+) when tested with the antiglobulin serum used for the test.

The titer values of the anti-Rh_0 serum used for these antiglobulin titrations were 6 units by the blocking, 80 units by the albumin-plasma, 160 units by the trypsinated cell and negative by the saline agglutination methods.

*The antiglobulin serums were diluted to meet the minimum requirements of the N. I. H. and then further diluted 1:8 with normal saline.

**Coating of trypsinated cells was not detected by the trypsinated cell indirect antiglobulin method when weak antiglobulin serums were used, and was detectable only by a slight increase in intensity of the reaction or an increase in titer of one tube, when antiglobulin serum was used that was of such titer that it just met or slightly exceeded the minimum requirements of the N. I. H. Higher titers were obtained when high titer antiglobulin serum was used.

concerned, symptoms do not, as far as is known, result from antibodies in extremely low titer. Yet, as Roberts[7] states "detection of very weakly sensitized cells may aid in the diagnosis of hemolytic anemia especially during a remission." In addition, a high titer antiglobulin serum might be of value in detecting sensitization produced by factors which are poor antigens and therefore produce antibodies in extremely low titer. It is possible that it might aid in disclosing antibodies previously undescribed. The trypsinated cell indirect antiglobulin method of titration, which was presented in a previous communication[5] as well as the standard indirect antiglobulin method of titration, possibly will not be adopted widely by clinical laboratories doing routine examinations. However, the results which the former test yields are of considerable theoretical interest. In describing the method, our intent has been to point out an additional method of investigating the nature and reaction of agglutinogens of human red cells.

SUMMARY

Cross-titrations of a single univalent anti-Rh_0 serum with six different antihuman globulin serums were carried out: Three of the antiglobulin serums were of commercial origin and three were not.

These tests showed that the titer of an anti-Rh_0 serum obtained by the indirect anti-human globulin method depended upon the potency of the antiglobulin serum used. Weak serums yield titer values lower than the trypsinated cell method and also render the trypsinated cell indirect antiglobulin method

insensitive. Commercial antiglobulin serums as presently released give titer values for an anti-Rh_0 serum approximately equal to or somewhat higher than those obtained by the albumin-plasma or by the trypsinated cell method, and therefore have little or no advantage. To make use of the maximum potentialities of the antiglobulin test, it should be carried out in *test tubes* with very potent serum. Such high titer serums are also needed for the trypsinated cell indirect antiglobulin method to detect antibodies present in a serum in extremely low titer and beyond the range of other methods.

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OBLITERATING TRACHEITIS, A COMPLICATION FOLLOWING ADMINISTRATION OF RADIOACTIVE IODINE

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WHILE using radioactive iodine, I^{131+},* in studies on the skeletal effects of hypo- and athyroidism, we lost a number of mice of strain Dba. These deaths were found to be caused by a radiation tracheitis and peritracheitis.

OBSERVATIONS

Male mice of the closely inbred strain Dba, four to five weeks old and weighing about 10 grams, were used. The animals kept on a standard diet of Purina Laboratory Chow and water ad libitum received a subcutaneous injection of 35 μc of I^{131+} per gram of body weight. This relatively large dose was intended to completely destroy all thyroid tissue and to prevent subsequent regeneration of thyroid remnants or of accessory thyroid glands. The animals did well during the first two months following the injection of I^{131+} and gained weight up to 20 grams. During the third and fourth months of observation, however, a number of mice lost weight and died. In many of the dead animals, autolysis was too far advanced to permit a satisfactory necropsy. Therefore, some mice that had lost weight were sacrificed. At necropsy, a small band of dense whitish tissue was seen crossing over the trachea and apparently exerting lateral pull on the latter. For microscopic studies, this segment of the trachea with the surrounding tissues was removed, fixed in Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Microscopic examination: Cross sections through the critical region of the trachea near the thyroid glands of these mice presented the following changes: the trachea was partly denuded of epithelium, and most of the submucosal glands were destroyed. The lumen of the trachea was narrowed or occluded by a vascular, partly loose, edematous, and partly dense fibrillar tissue containing fibroblasts, mononuclear and polymorphonuclear leukocytes with an admixture of fibrin and nuclear debris in the central portions (Fig. 1). In some areas, the narrowed tracheal lumen had been relined by pseudostratified or low cuboidal epithelium (Fig. 2). The perichondrium of the tracheal cartilage was thick, the cartilage cells were swollen, and showed pyknosis of nuclei and karyorrhexis. In other places, the cartilage was eroded

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Fig. 1. Fig. 3.

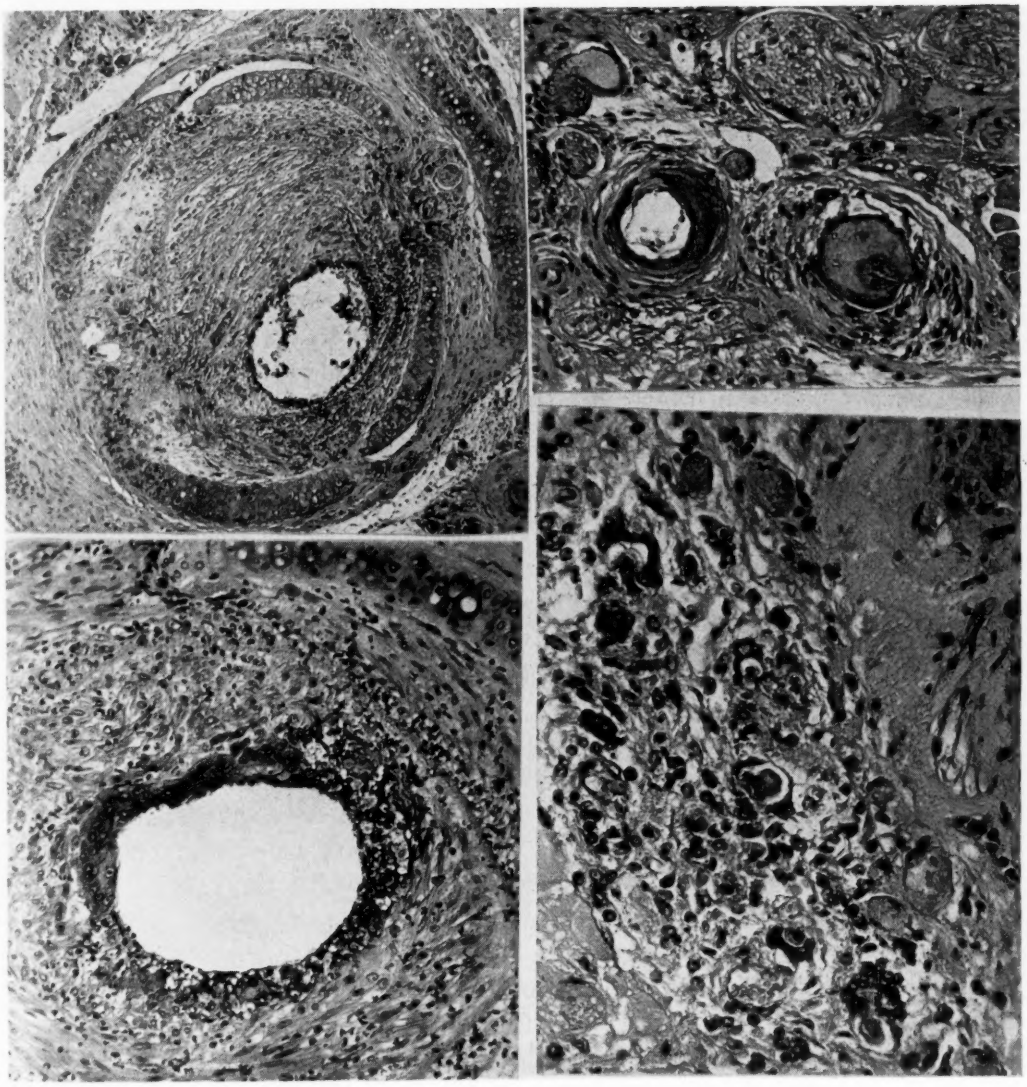

Fig. 2. Fig. 4.

Fig. 1.—Cross section through the trachea showing the lumen obstructed by fibrous tissue which leaves only a narrow airway open. A chronic inflammatory process is seen in the peritracheal tissue. (Magnification ×95.)

Fig. 2.—Partial relining of the narrowed airway by pseudostratified epithelium; marked leukocytic infiltration of the newly formed intratracheal connective tissue. (Magnification ×230.)

Fig. 3.—Thickened, hyalinized and calcified arteries in the vicinity of the original thyroid site. There is perivascular fibrosis. (Magnification ×255.)

Fig. 4.—Foreign body reaction with the formation of multi-nucleated giant cells in the peritracheal connective tissue. (Magnification ×400.)

by the granulation tissue that had developed within the lumen of the trachea. The peritracheal tissue was diffusely edematous and infiltrated by fibroblasts, mononuclear and polymorphonuclear leukocytes. The capillaries were engorged with blood. The walls of the vessels were markedly thickened and incrustated with calcium; the lumina of some vessels were obstructed by round or ovoid calcified bodies surrounded by mononuclears (Fig. 3). The adventitia was increased in amount, and contained fibroblasts, mononuclears, and polymorphs. Throughout the peritracheal tissue, there were small foci of necrosis which had undergone calcification. Such areas were encapsulated by connective tissue containing a good many multinucleated foreign body giant cells which were also present near the calcified areas (Fig. 4). Some muscle cells showed swelling, degranulation of the cytoplasm, and pyknosis of nuclei with polymorphonuclear and mononuclear cell infiltration.

The thyroid tissue was in some cases destroyed, while in others small colloid-containing follicles, or distorted foci of follicular epithelium, could still be identified.

DISCUSSION

In male mice of strain Dba receiving 35 μc of I^{131} per gram body weight, tracheal lesions were observed within two to three months after the beginning of the treatment. These tracheal changes may be classified as a proliferative endo- and peritracheitis associated with thickening and calcification of the regional arteries. These findings constitute the typical microscopic picture of radiation injury.

Stenosing granulomatous lesions in the trachea have been observed in patients subsequent to x-ray treatment of the thyroid glands. These changes were late effects, and were found from one to seven years subsequent to radiation.[1] Moreover, injury to the trachea has been noted in hyperthyroid patients treated with high doses of radioactive iodine.[2, 3]

In mice, the tracheal epithelium has been shown to be extremely sensitive to radiant energy accumulated in the thyroid gland. After injections of high dosages of I^{131}, regressive changes in the tracheal mucosa were seen within one day. Usually, within one week, regeneration occurred, and the regenerative processes were associated with squamous metaplasia of the injured epithelium. If, however, doses exceeding 300 μc were injected, fibrous tumors were observed in the trachea several months following the administration of I^{131}.[4]

We did not find tumors in the trachea of mice treated with I^{131}. However the possibility cannot be excluded that intratracheal tumors may develop in animals surviving for long periods of time.

It may be noteworthy that even with high doses of I^{131}, destruction of thyroid tissue was not complete under the conditions of our experiment and that after four months foci of distorted acini were occasionally observed.

SUMMARY

In male mice of strain Dba receiving at the age of one month 35 μc of I^{131} per gram body weight, a fatal, obstructive endo- and peritracheitis were observed three to four months after beginning of the treatment. The lesion showed the typical microscopic features of radiation reaction.

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EFFECT OF SODIUM AND ZIRCONIUM CITRATES ON DISTRIBUTION AND EXCRETION OF INJECTED RADIOLEAD

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THE possibility of chemical interaction in vivo between lead and administered drugs has been the basis of numerous attempts to treat lead poisoning.[1-4] One approach has involved the use of compounds forming soluble complex ions with lead such as ascorbic acid,[5] tartaric acid,[1] and citric acid.[6-9] Of these compounds citric acid has produced the most favorable clinical response, presumably because it forms a particularly stable and diffusible complex ion with lead.[10] The effects of added citrates on the metabolism of lead in the living animal have not been investigated in any detail. In this paper we are reporting our studies of the effects of sodium and zirconium citrates on the blood level, tissue distribution, and excretion of radiolead, Pb^{210}, with and without the addition of carrier lead. Zirconium was used because it was thought that the colloidal aggregates formed by the Zr in the blood would adsorb the circulating Pb and cause an increased excretion and a redistribution of the Pb in a manner similar to that proposed for the action of Zr salts on the metabolism of Pu and the rare earth elements.[11, 12]

EXPERIMENTAL

Female Sprague-Dawley rats weighing 220 to 270 Gm. were injected intravenously with a carrier-free solution of Pb^{210} at a pH of 6 to 7, and containing less than 1 μg of total lead. The solution was prepared by mixing a small volume of Pb^{210} contained in dilute HCl with either 0.16M sodium acetate or 0.16M sodium chloride. Each rat received from 0.2-0.6 μc. of Pb^{210}, or about 30,000 to 100,000 counts per minute under our counting conditions. In other experiments lead acetate was added as carrier in such amounts that each rat received 1.03 mg. of lead.

The sodium citrate solutions were prepared by dissolving a weighed amount of reagent grade salt in freshly boiled distilled water. The zirconium citrate solutions were prepared in the manner described previously.[11] The citrate solutions were administered intravenously or intraperitoneally at stated times following the Pb injections.

The rats were kept in individual metabolism cages and the urine and feces collected separately. After the animals were sacrificed by intraperitoneal injection of Nembutal, several tissues were removed for analysis. Terminal blood samples (1 to 3 ml.) were obtained by cardiac puncture, while all other blood samples (0.2 to 0.3 ml.) were obtained from a lateral tail vein. Volumes were measured in a hypodermic syringe. Plasma was separated from the cells by centrifuging the fresh blood sample for thirty minutes at approximately 3,000 revolutions per minute before removing the supernatant plasma with a pipette. The cells were then rinsed twice with isotonic saline and centrifuged ten minutes between rinses. The saline was added to the plasma fraction. The hematocrit for all rats was taken to be 50, the approximate mean value of our determinations. Total blood volume was taken to be 6.06 per cent of the body weight.[13]

Tissues were wet ashed with concentrated nitric acid and made up to 100 ml. with dilute HNO_3. Measured aliquots were placed in 10 ml. flat-bottomed porcelain counting capsules and dried by overhead heating with an infrared bulb. In the case of small tissues such as the 0.2 to 0.3 ml. volumes of blood, the entire sample was wet ashed directly in a counting

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capsule. The activity in a given organ was compared with that in a standard. This was prepared by adding an aliquot from the dummy injection solution (made by placing the identical amount of injection solution given each rat into a volumetric flask and adjusting to volume with HNO_3) to an inactive organ. It was then prepared for counting as described above. The β- radioactivity of the samples was measured by counting under an end mica-window counter tube. An aluminum filter of 13.5 mg. per square centimeter was used to screen out alpha-emitting isotopes. The activity measured was that of the Bi^{210} daughter of Pb^{210}; hence at least six half-lives (thirty days) were allowed to elapse before the samples were counted.

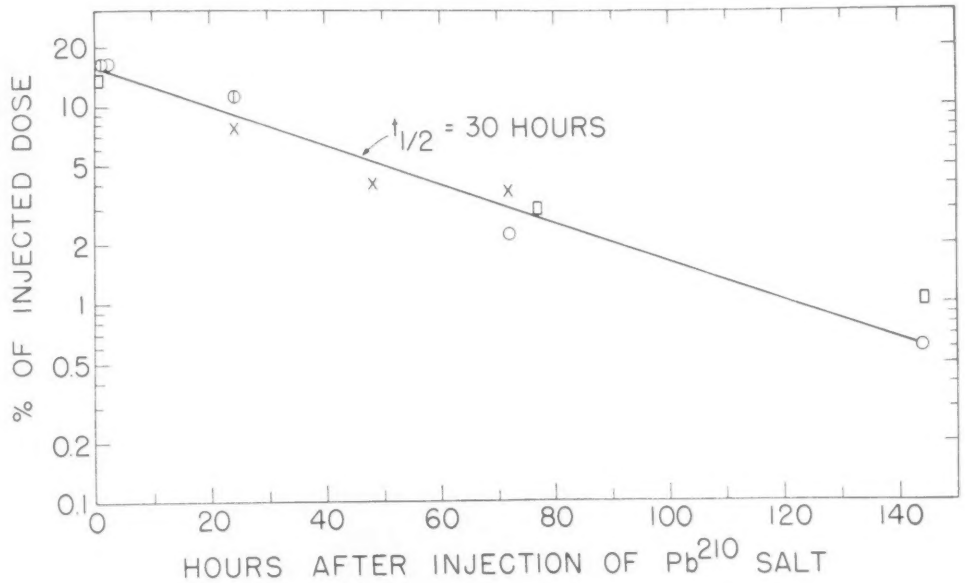

Fig. 1.—Rate of disappearance of intravenously injected carrier-free Pb^{210} salts from the whole blood (essentially the cellular fraction) of rats. Per cent of injected dose of Pb^{210} in the total blood volume is plotted against time after injection on a semi-logarithmic plot. The symbols ○, ×, and ⊕ represent rats of three different experiments, all receiving the Pb^{210} in a 0.16M sodium acetate solution, while the symbol □ represents rats receiving Pb^{210} in a 0.16M solution of sodium chloride. Values from twenty-two rats are included in the figure. Each symbol is a mean value from an average of two rats.

RESULTS

Blood Disappearance of Pb^{210} After Citrate Administration.—In preliminary tests we confirmed early work[14, 15] showing that within a few minutes after the injection of *small* amounts (~1 mg. per 100 ml. blood) of soluble lead salts nearly all of the circulating lead is in the blood cells (red plus white). We then determined the half-time of disappearance of Pb^{210} from the cellular fraction (actually the whole blood was analyzed) after the injection of carrier-free Pb^{210}. The data given in Fig. 1 show that the tracer Pb^{210} disappeared exponentially with a half-time of about thirty hours.

To determine the effect of sodium and zirconium citrates on the blood disappearance rate of Pb^{210}, thirteen rats were injected with either Pb^{210} acetate or chloride. Thirty minutes later they were divided into groups of two or more rats, and the treated rats were given either 4 ml. of a 3 per cent solution of

TABLE I. EFFECT OF SODIUM AND ZIRCONIUM CITRATE ADMINISTRATION ON THE DISTRIBUTION AND EXCRETION OF INTRAVENOUSLY INJECTED CARRIER-FREE Pb^{210} ACETATE BY RATS*

(RESULTS ARE EXPRESSED AS PER CENT OF INJECTED DOSE OF Pb^{210}. TWO OR THREE RATS WERE SACRIFICED AT EACH POINT)

	CONTROL RATS				TREATED RATS			
					ZIRCONIUM CITRATE		SODIUM CITRATE	
					30 MIN. AFTER Pb	3 DAYS AFTER Pb	30 MIN. AFTER Pb	3 DAYS AFTER Pb
TIME SACRIFICED	30 MIN.	1 DAY	3 DAYS	6 DAYS	6 DAYS	6 DAYS	6 DAYS	6 DAYS
TISSUE	%	%	%	%	%	%	%	%
Carcass	44.7 ± 1.4	—	58.3	44.0 ± 3.7	40.1 ± 0.3	50.4 ± 2.9	45.1 ± 1.7	45.6 ± 0.6
Femurs	2.3 ± 0.1	3.5 ± 0.1	4.0 ± 0.1	3.7 ± 0.1	3.6 ± 0.0	4.1 ± 0.4	4.3 ± 0.1	4.0 ± 0.2
Liver	14.5 ± 0.2	7.8 ± 0.3	6.1 ± 0.2	2.3	2.2 ± 0.3	2.2 ± 0.1	2.0 ± 0.5	1.9 ± 0.3
Kidneys	14.1 ± 1.7	10.6 ± 0.5	7.4 ± 0.2	3.3 ± 0.4	2.3 ± 0.3	3.9 ± 0.1	2.7 ± 0.4	2.8 ± 0.1
Spleen	0.2 ± 0.0	0.3	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
Total blood	17.2 ± 2.0	10.0 ± 1.0	2.3 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	1.1 ± 0.4	0.6 ± 0.2	0.7 ± 0.0
Urine—day 1				11.0 ± 2.0	12.9 ± 1.2	8.2 ± 0.6	9.6 ± 1.4	11.1 ± 0.1
day 2,3				5.4 ± 0.1	5.3 ± 0.2	5.3 ± 0.5	4.5 ± 0.8	4.9 ± 0.8
day 4				1.7 ± 0.3	1.7 ± 0.0	1.9 ± 0.3	1.2 ± 0.1	1.6 ± 0.2
day 5,6				2.3 ± 0.4	1.6 ± 0.0	2.1 ± 0.2	1.8 ± 0.0	1.8 ± 0.1
Total urine				20.4	21.5	17.5	17.1	19.4
Feces—day 1				4.9 ± 3.2	5.3 ± 1.4	9.8 ± 2.5	11.5 ± 1.6	6.3 ± 0.1
day 2,3				14.5 ± 4.0	15.5 ± 3.0	10.8 ± 2.0	8.6 ± 0.8	11.1 ± 0.3
day 4				2.0 ± 0.1	2.5 ± 0.5	2.3 ± 0.2	1.9 ± 0.1	1.8 ± 0.0
day 5,6				2.4 ± 0.2	2.9 ± 0.6	2.8 ± 0.4	2.6 ± 0.5	2.8 ± 0.1
Total feces				23.8	26.2	25.7	24.6	22.0
Total excreted				44.2	47.7	43.2	41.7	41.4
Mean recovery				98.2	96.5	105.0	96.5	96.4

*The rats were injected intravenously with 0.5 ml. of a 0.16M sodium acetate solution containing carrier-free Pb^{210} equivalent to about 30,000 c.p.m. under our counting conditions. Either thirty minutes or three days later treated rats were injected intravenously with either 1.0 c.c. of a 3 per cent sodium citrate solution or with 1.0 c.c. of a zirconium citrate solution containing 14.3 mg. as Zr per cubic centimeter. The figure given is a mean, followed by the standard error.

sodium citrate* intraperitoneally, 1 ml. of the same solution intravenously, or 50 mg. of Zr in the citrate form intraperitoneally. Altogether, more than thirty blood samples were withdrawn from the tail veins at regular intervals for a two-hour period beginning one minute after treatment. Neither sodium nor zirconium citrate had any effect greater than the normal experimental variations on the Pb^{210} disappearance rate. Subsequently, we found that even the injection of Pb^{210} in a 1½ per cent sodium citrate solution did not affect the rate of Pb^{210} disappearance from the blood.

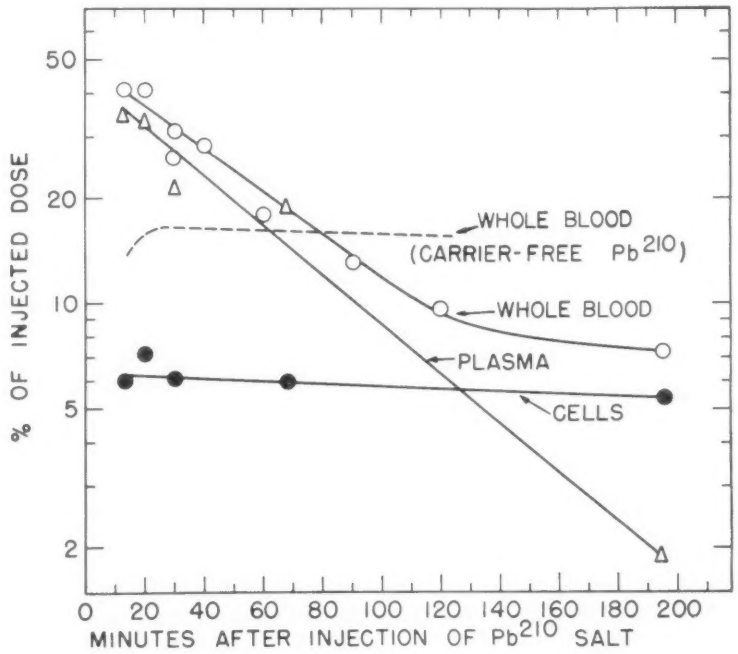

Fig. 2.—Rates of disappearance of intravenously injected Pb^{210} with added carrier from the components of the blood in rats. Each rat received an intravenous injection of 1.03 mg. of Pb as lead acetate with a tracer amount of Pb^{210}. Each symbol represents the amount of activity in the total volume of circulating whole blood or stated blood fraction. Blood cell and plasma analyses were made from measured aliquots of cardiac blood taken at sacrifice, while the whole blood sample was usually taken from the tail vein.

○—whole blood
△—plasma fraction
●—blood cell fraction

The dashed line represents the activity in the blood (essentially the cells) when carrier-free Pb^{210} is injected intravenously.

We then investigated whether citrate administration would affect the disappearance of Pb^{210} from the plasma. Rats were injected intravenously with 1.03 mg. of Pb with tracer Pb^{210}. Thirty minutes later the plasma contained 22 per cent of the injected dose, and the cells only 6 per cent (Fig. 2). This plasma fraction disappeared rapidly with an approximate half-time of disappearance of only forty-two minutes (Fig. 2). In an experiment similar to that described above in the carrier-free series, rats injected with Pb^{210} plus the carrier were given either 3.0 ml. of a 3 per cent sodium citrate solution or 37.5 mg. of Zr in

*The normal citric acid concentration in the blood of the rats used in these experiments varies from about 15 to 35 μg per ml. of whole blood.[16]

3.0 ml. of zirconium citrate solution intraperitoneally after thirty minutes. Even under these conditions, when most of the circulating lead was in the plasma, the citrate solutions had no effect on the immediate rates of disappearance of Pb^{210} from the blood.

Excretion and Tissue Distribution of Pb^{210} After Citrate Administration.—Table I shows that intravenous administration of sodium or zirconium citrate either thirty minutes or three days after injection of *carrier-free* Pb^{210} acetate had no effect on either the urinary or fecal excretion of Pb^{210}, and no significant effect on the soft tissue distribution. The amount of sodium citrate given was approximately equivalent to 300 ml. of a 3 per cent solution in the human being.

Similar results were obtained in several other experiments, in which carrier-free Pb^{210} chloride as well as acetate was used, even when the dosage of zirconium or sodium citrate was increased by a factor of four.

TABLE II. EFFECT OF SODIUM AND ZIRCONIUM CITRATE ADMINISTRATION ON THE DISTRIBUTION AND EXCRETION OF Pb^{210} IN RATS RECEIVING 1.03 MG. OF Pb PER RAT*

(RESULTS ARE EXPRESSED AS PER CENT OF INJECTED DOSE OF Pb^{210}. THREE RATS WERE SACRIFICED AT EACH POINT)

	CONTROL RATS		TREATED RATS	
			ZIRCONIUM CITRATE	SODIUM CITRATE
TIME SACRIFICED	30 MIN.	1 DAY	1 DAY	1 DAY
TISSUE	%	%	%	%
Femurs	0.5 ± 0.1	2.2 ± 0.1	2.0 ± 0.2	2.1 ± 0.2
Liver	60.4 ± 4.0	14.3 ± 2.8	17.9 ± 2.3	17.7 ± 1.8
Kidneys	3.3 ± 1.0	17.9 ± 0.9	7.7 ± 0.9	13.4 ± 0.9
Spleen	1.4 ± 0.1	0.7 ± 0.2	1.0 ± 0.3	0.8 ± 0.0
Total blood	31.0 ± 0.0	1.2 ± 0.1	2.1 ± 0.1	1.5 ± 0.2
Urine	—	3.2 ± 0.5	11.5 ± 2.0	3.2 ± 1.0
Feces	—	12.8 ± 3.6	9.4 ± 1.6	13.7 ± 2.6

*The rats were injected intravenously with 0.55 ml. of a 0.16M sodium acetate solution containing 1.03 mg. of Pb as Pb acetate and 100,000 c.p.m. of Pb^{210}. Thirty minutes later the treated rats were injected intraperitoneally with 3.0 ml. of a 3 per cent sodium citrate solution or 3.0 ml. of a zirconium citrate solution containing 12.5 mg. as Zr per milliliter. Each figure is a mean, followed by the standard error of the mean.

In those experiments in which the rats were injected with 1.03 mg. of carrier Pb, sodium citrate administration was again without any significant effect on Pb^{210} distribution or excretion (Table II). Zirconium administration, however, resulted in an increase of urinary excretion of Pb^{210} from 3.2 to 11.5 per cent of the injected dose within the first twenty-four hours. The increased urinary Pb appears to be derived from the kidneys (Table II). These results confirmed those of an earlier experiment run under almost identical conditions.

It is of interest to compare the twenty-four hour distribution of Pb^{210} in the rats receiving carrier-free Pb^{210} with that in those receiving the added carrier (Tables I and II). The former rats excreted a greater per cent of the dose of Pb in the urine and retained a greater fraction of the injected Pb in the femurs and whole blood, but less in the spleen, kidneys, and liver. These differences in distribution are probably a reflection of the size of the colloidal aggregates of lead phosphate[1] formed in the blood, since it is to be expected that the larger particles, formed with the addition of the carrier, would concentrate in the reticulo-endothelial system.

DISCUSSION

The results described in this paper indicate that the citrate ion has little or no effect on the immediate blood disappearance, organ distribution, or excretion of lead in rats. Sodium citrate treatments of cases of lead poisoning in human beings have generally been reported to cause increases in Pb excretion[6, 9] and a decline in blood Pb levels,[7] as well as a marked alleviation of the symptoms. The recent report of Hardy and associates,[17] however, does not show any consistent effect of sodium citrate on the urinary excretion of Pb (the fecal output, which might be significant, was not studied). It is conceivable that in the case of human beings three or four treatments a day might elevate the blood citrate level for longer periods than the two- to three-hour increase found after one injection,[18] and thereby might increase the blood disappearance and excretion of Pb. In this respect the rate of formation of the lead citrate complex ion from the insoluble lead compounds in the blood may be a factor to be considered in the treatment of lead poisoning.

The effects of sodium citrate on plumbism may be caused by its alkalizing effect or by the citrate ion itself, which forms a complex with lead. The ability of the citrate ion to increase the fraction of diffusible lead in plasma and serum in vitro has been demonstrated.[19] Our data do not rule out the possibility that complex ion formation between plasma lead and citrate occurs in vivo.

If, as many cases of lead poisoning have indicated, the clinical symptoms are related to the plasma levels,[3, 20] the relief offered by many drugs might be explained in terms of the rapid disappearance rate of Pb from the plasma in addition to, or instead of, in terms of complex ion formation. For example, if alkalizing agents (such as sodium citrate) or acidifying agents diminish the rate of transfer of Pb from the skeleton into the circulation, the natural, rapid disappearance of Pb from the plasma (Fig. 2) should result in an alleviation of the symptoms of Pb poisoning.

The increase in Pb excretion in rats following Zr treatment may be worth further investigation to determine the practicality of its application to the treatment of Pb poisoning in human beings.

SUMMARY

Twenty-five minutes after intravenous injection of *carrier-free* Pb^{210} into rats, approximately 10 per cent of the injected dose of Pb^{210} was in the blood (essentially the cellular fraction), from which it disappeared with a half-time of 30 hours. After the same interval following injection with carrier Pb, only 6 per cent of the injected Pb^{210} was in the blood cells and approximately 22 per cent in the plasma. The Pb in the plasma fraction left rapidly, less than 2 per cent of the dose remaining after three hours.

Sodium or zirconium citrate injected after *carrier-free* Pb^{210} did not affect the rate of disappearance of Pb^{210} from the blood, the tissue distribution, or the fraction excreted.

In rats injected with *carrier* amounts of Pb labeled with Pb^{210}, zirconium citrate did not affect the immediate rate of disappearance of Pb^{210} from the

blood, but it caused over a threefold increase in the Pb^{210} excreted during the first twenty-four hours and a decrease in the kidney concentration. The sodium citrate was without effect on blood disappearance rate, tissue distribution, or excretion of the Pb^{210}.

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THE PROPHYLAXIS OF MALARIA AND AMEBIASIS WITH MILIBIS-ARALEN*

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MALARIA and amebiasis continue to be responsible for an immense amount of sickness and suffering in all parts of the world. This is perhaps especially true in tropical and subtropical areas, but is by no means limited to them. The importance of these two diseases to North Americans is twofold: (1) they both occur in the United States (malaria now infrequently, but amebiasis in from 5.0 to 10.0 per cent of the population), and (2) they both occur with greater frequency in locations to which American troops may be sent in the future. Thus there is good reason to continue the search for more effective antimalarial and amebicidal drugs, both for the treatment of the clinical case and for mass prophylaxis.

Foremost of the available antimalarial drugs are two very effective 4-aminoquinoline compounds, Chloroquine (Aralen^R) and Camoquin^R. And currently foremost among amebicidal drugs are the antibiotics aureomycin, bacitracin, and terramycin, and the bismuth glycolylarsanilate called Milibis^R. It has seemed desirable, for long term prophylactic use, to combine an antimalarial substance and an amebicidal drug in a single tablet. Such a combination of Aralen^R and Milibis^R has been prepared and its use is the subject of this paper.

Aralen^R has the formula (4-diethylamino-1-methylbutylamino) quinoline. For oral use it is given in the form of the diphosphate, a white crystalline powder readily soluble in water at an acid pH level. Effectual treatment of either falciparum or vivax malaria is accomplished with only 2.0 Gm. (8 tablets) or less, in a twenty-four hour period; and effective prophylaxis is maintained with approximately 500 mg. (2 tablets) weekly. Its incidence of side reactions is low and includes transient mild headache, mild nausea, occasional pruritus, and disturbance of visual accommodation.

The remarkably beneficial effect of Aralen^R on extraintestinal amebiasis is also to be emphasized, particularly since our current concept of amebiasis is that systemic involvement occurs fairly early in many persons with intestinal infections. Many clinicians have substituted Aralen^R for emetine in the treatment of extraintestinal amebiasis. The regimen recommended by Conan is generally followed. This consists of a priming dose of 1.0 Gm. daily for two days, followed by 0.5 Gm. daily for two to three weeks.

Milibis is bismuthoxy-para-N-glycolylarsanilate, a bismuth derivative of p-N-glycolylarsanilic acid. It contains 15.01 per cent of arsenic and 41.88 per cent of bismuth. Effective in vivo animal amebicidal activity was demonstrated

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*The Milibis-Aralen Tablets were supplied by Winthrop-Stearns, Inc., New York, N. Y.

by Hauer[1] and by Dennis, Berberian, and Hansen.[2] Berberian[3] then treated seventy-two cases of amebiasis with varying dosages of Milibis[R] with good results, and showed at the same time that there was no significant improvement in the relapse rate when either chiniofon or bismuth subgallate was combined with Milibis[R]. During the initial period of clinical investigation, Milibis[R] was used in 1,186 cases of amebiasis[4] with good results; in one study, a total of 381 (82.6 per cent) of 461 cases followed after therapy, were parasitologically "cured" with Milibis[R] alone, after one to four courses of the drug. As a result of these and other[5-11] studies, the recommended average adult dose of Milibis[R] in intestinal amebiasis is 0.5 Gm. three times a day for seven days. A satisfactory dosage schedule for the prophylaxis of amebiasis is not known.

The Milibis-Aralen tablets which were used in this study of mass prophylaxis each contain 250 mg. of Milibis[R] and 75 mg. of Aralen[R].

MATERIAL AND METHODS

The present study was carried out in a well-circumscribed banana labor camp located two miles from the Tela Railroad Company (United Fruit Co.) headquarters at La Lima, Honduras. The camp has a population of approximately 500 men, women, and children living in twenty-two subdivided buildings. The water supply and sanitary facilities are exactly the same throughout the camp.

Initially, stool and blood surveys were carried out in the entire camp. Single, casually passed stool specimens were examined for *E. histolytica* directly, after staining with D'Antoni's Iodine, and then again after zinc sulfate flotation. The percentage of positive stool specimens was 34.9 per cent. Twenty-eight per cent had cysts, 5.4 per cent had cysts and trophozoites, 2.4 per cent had trophozoites only. None of the initial thick blood films were positive for *Plasmodia*.

Next, alternate buildings were selected for treatment and control. In this way, there were 201 individuals in "Group I" with an incidence of 36 per cent positive stools; this group was chosen for treatment with the Milibis-Aralen tablets for a twelve-week period according to the dosage scale given in Table I. The compound was administered by roster, i.e., each individual stood in turn before the dispenser and swallowed the tablets under direct observation. There were 209 individuals in "Group II" with an incidence of 34 per cent positive stools; this was designated the control group.

During the twelve-week period of the study, record was kept of morbidity in both the treatment and the control groups, particularly of malaria and of clinically evident amebiasis. Individuals receiving Milibis-Aralen were questioned regularly about the occurrence of side reactions. At the close of the twelve-week period, the stool and blood surveys were again carried out. The procedure was identical to the initial survey.

TABLE I. DOSAGE SCALE OF MILIBIS-ARALEN*

AGE GROUP	NUMBER OF INDIVIDUALS TREATED	DOSAGE (A SINGLE DOSE ON 2 CONSECUTIVE DAYS EACH WEEK FOR 12 WEEKS)	TOTAL NUMBER OF TABLETS IN 12-WEEK PERIOD	AMOUNT OF MILIBIS IN 12-WEEK PERIOD	AMOUNT OF ARALEN IN 12-WEEK PERIOD
Less than 1 year	12	½ tablet	12	3.0 Gm.	0.8 Gm.
1 to 3 years	31	1 tablet	24	6.0 Gm.	1.6 Gm.
4 to 6 years	26	2 tablets	48	12.0 Gm.	3.2 Gm.
More than 6 years	132	3 tablets	72	18.0 Gm.	4.8 Gm.

*Each tablet contains 250 mg. of Milibis and 75 mg. of Aralen.

RESULTS AND COMMENTS

The results of the Milibis-Aralen treatment are shown by the comparison of initial and final stool surveys in Table II and by a consideration of clinical cases of malaria and amebiasis occurring between the two surveys.

Table II is self explanatory; the fall in the incidence of positive stools among the treated group is striking. The inclusion of separate figures for the cysts and trophozoites of *E. histolytica* in Table II and in the text merits brief comment. This has been done for the completeness of the record and to indicate to some degree the severity of the disease process, inasmuch as trophozoite forms are passed only when diarrhea is marked or dysentery occurs. It should be emphasized that there is no intent to speak of the treatment of cysts, as over against the treatment of trophozoites. One does not treat cysts. Trophozoite forms are solely responsible for pathogenesis in amebiasis; cystic forms cause no pathogenesis. It is because cystic forms develop from the trophozoites that their presence or absence is used as an index of success or failure of the therapeutic procedure aimed at the trophozoite. This fact needs repetition because it seems to be frequently misunderstood.

During the twelve-week period there were no cases of clinical amebiasis or clinical malaria in the treated group. However, from the nontreated group during the same time period, there were twelve hospital admissions for symptomatic intestinal amebiasis, one for hepatic amebiasis, eight for falciparum malaria, and seven for vivax malaria.

Side effects from the Milibis-Aralen tablets were almost negligible. In only two instances were there complaints during the course of therapy.

A 40-year-old woman mentioned severe low abdominal pains several times, but there was complete disappearance of the pain without discontinuance of the Milibis-Aralen. A 24-year-old man developed mild, bilateral nerve-type deafness after the eighth week of treatment which did not disappear even after discontinuance of the drug; this may or may not have been related to the medication.

TABLE II. RESULTS OF INITIAL AND FINAL STOOL SURVEYS AMONG THE GROUP TREATED WITH MILIBIS-ARALEN AND AMONG THE CONTROL GROUP

	GROUP I (TREATED)	GROUP II (CONTROL)
Number of Individuals	201	209
Per cent of positive stools before study	35.8	33.9
a. Cysts only	28.8	25.3
b. Trophozoites only	3.0	1.9
c. Cysts and trophozoites	4.0	6.7
Number of Individuals Included in Second Survey 12 Weeks Later	188*	190†
Per cent of positive stools in second survey	3.4	28.9
a. Cysts only	3.4	23.7
b. Trophozoites only	–	2.1
c. Cysts and trophozoites	–	3.1

*Of the thirteen individuals from the treated group who did not have a stool examination in the second survey, five, or 38.5 per cent, had had positive stools in the initial examination. The percentage, therefore, was approximately the same as that for the entire group and the loss of these thirteen cases does not alter the results.

†Of the nineteen individuals from the untreated group who did not have a stool examination in the second survey, eight, or 42.1 per cent, had had positive stools in the initial examination. The percentage is, therefore, a little higher than that for the entire group, but not so much that it significantly alters the results.

SUMMARY

Tablets containing Milibis[R] and Aralen[R] acted as an efficient prophylactic agent against both amebiasis and malaria when administered in small doses on two consecutive days each week. In areas where the two diseases are endemic, this drug combination will prove to be a valuable compound.

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THE EFFECTS OF CORTISONE ON BACTERIAL INFECTION

Group A Hemolytic Streptococcal Infection in Rabbits

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With the Technical Assistance of Joan Floyd

IN THE course of a study of the effects of cortisone and ACTH on various types of tissue damage, it was noted that the response of rabbits to intracutaneously or intravenously injected meningococcal endotoxin was markedly altered when the rabbits were treated with large doses of cortisone. After intracutaneous injection, numerous small hemorrhages appeared at the injected skin site[1]; after intravenous injection of toxin, bilateral cortical necrosis of the kidneys occurred.[2] Such lesions did not occur in rabbits which were not treated with cortisone.

The enhancement of tissue damage by bacterial products prompted an investigation into the effects of cortisone on experimental infection with various types of living microorganisms. As one approach to the problem, rabbits were treated for several days with cortisone and then infected intracutaneously with various strains of group A hemolytic streptococci. It was found that such treatment resulted in extensive streptococcal bacteremia and death in a high percentage of animals, while untreated control rabbits survived without bacteremia. A preliminary account of these experiments has been given elsewhere.[3, 4]

Other investigators have reported the enhancement by cortisone of the extent of infection with various unrelated agents, including pneumococci,[5] tubercle bacilli,[6] spirochetes,[7] *Brucella*,[8] and poliomyelitis,[9] influenza, mumps,[10] and Coxsackie viruses.[11] No satisfactory explanation is available for these remarkable effects of cortisone. Obviously, a further knowledge of the mechanisms involved would contribute much to our understanding of fundamental problems of resistance and susceptibility to infection.

The present report is concerned with a description of the conditions required for the production of fatal streptococcal septicemia by the administration of cortisone or ACTH. Certain hematologic, immunologic, and pathologic events which occur during infection will be considered, and the effect of prior immunization with streptococci on the incidence of septicemia and death will be described.

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MATERIAL AND METHODS

Streptococci.—Four different strains of group A hemolytic streptococci were employed in these experiments. These were designated as follows: MA—type 1, ASO—type 3, I—type 17, and D—type 24. These strains were supplied through the kindness of Dr. A. T. Wilson, of the Alfred I. du Pont Institute, Dr. George Murphy, of The Rockefeller Institute for Medical Research, and Dr. Charles Rammelkamp, of the Western Reserve University School of Medicine. All strains were received in this laboratory as lyophilized cultures and were maintained on rabbit blood agar, with occasional passages in mice. Suspensions of streptococci for the infection of rabbits were prepared by growing cultures for fifteen hours in Todd-Hewitt broth and making serial tenfold dilutions in sterile broth. An estimation of the number of organisms used for infection was obtained by making duplicate pour plates and counting colonies after forty-eight hours of incubation.

Rabbits.—Hybrid male and female rabbits, weighing approximately 2 kilograms, were obtained from local breeding sources. They were maintained on a diet of Rockland rabbit pellets supplemented by greens. Prior to intracutaneous injections of streptococci, the hair over the back and lateral abdominal wall was clipped closely. All bleedings for cultures and serologic or hematologic studies were made from a marginal ear vein, under aseptic precautions.

Cortisone acetate (11-dehydro-17-hydroxycorticosterone-21-acetate) was obtained through the generosity of Merck and Company. Suspensions of the crystalline material were prepared in sterile physiologic saline in a concentration of 25 mg. per milliliter. All injections were given intramuscularly in the thigh. The dosage and schedule of administration will be described below.

ACTH.—Adrenocorticotropic hormone, derived from hog pituitary, was obtained in lyophilized form from the Armour Laboratories. The material was dissolved in sterile physiologic saline in a concentration of 10 mg. per milliliter, and injections were made in the thigh muscles.

Blood Cultures.—Blood was collected from the marginal ear vein in amounts of 3 to 5 ml. in sterile tubes containing 3 per cent sodium citrate. Pour plates were made by mixing 1 ml. of whole blood with beef heart infusion agar, or, when colony counts were required, combining smaller amounts of blood with normal rabbit blood and mixing with infusion agar. Approximately 4 ml. of whole blood were also added to 35 to 50 ml. of Todd-Hewitt broth. The pour plates and broth cultures were examined during a period of seventy-two hours at 37° C.

Serum for antibody studies and other serologic test was stored at −20° C. immediately after being obtained.

Antistreptolysin O determinations were made by the method of Swift and Hodge.[12] Standard immune serum of known titer was obtained through the courtesy of Dr. Charles Rammelkamp, and a control was run with each set of titrations. In most instances serial samples of serum from individual rabbits were tested together on the same day. Titers were expressed as the logarithm of the reciprocal of serum dilutions; titrations were begun at a dilution of 1 to 50 (log unit 1.7) with increments of 0.1 logarithmic unit.

Blood specimens for cell counts and sedimentation rates were collected in tubes containing dry potassium and ammonium oxalate. Wintrobe tubes were used for sedimentation rates, which were read at one hour, and the tubes were then centrifuged at 2,000 r.p.m. for thirty minutes for hematocrit determinations.

Pathologic Studies.—Autopsies were performed immediately after death wherever possible, and in no instance to be reported were autopsies delayed for longer than twenty minutes after death. Living animals were sacrificed where indicated by a blow to the head. Representative samples of organs were fixed in 10 per cent formalin and stained with hematoxylin-eosin and Giemsa stains.

EXPERIMENTAL

I. Intracutaneous Infection in Cortisone-Treated Rabbits.—Rabbits were infected intracutaneously by an injection, in the skin of the flank, of 0.25 ml. of

TABLE I. INCIDENCE OF BACTEREMIA AND DEATH FOLLOWING INTRACUTANEOUS INJECTION OF GROUP A HEMOLYTIC STREPTOCOCCI IN CORTISONE-TREATED AND UNTREATED RABBITS

STREPTOCOCCUS STRAIN	CORTISONE TREATMENT* MG./DAY	NO. OF STREPTOCOCCI INJECTED†	NO. OF RABBITS	NO. WITH BACTEREMIA‡	NO. DEAD	DAY OF DEATH§	MORTALITY PER CENT
MA-1	12	130	8	8	8	3, 5, 7, 8, 8, 10, 11, 14	100
	12	105	10	10	9	2, 3, 4, 4, 5, 6, 7, 8, 10	90
	12	106	10	10	10	2, 2, 2, 2, 3, 4, 4, 4, 4, 5	100
	None	105	8	0	0		0
	None	106	10	0	0		0
	None	109	12	2	1	12	8
ASO-3	12	102	8	8	8	2, 2, 2, 3, 3, 4, 4, 4	100
	12	104	10	10	10	2, 2, 2, 2, 2, 3, 3, 3, 4, 8, 10	100
	12	107	8	8	8	2, 2, 3, 3, 4, 4, 5, 8	100
	6	104	9	8	7	6, 6, 7, 9, 12, 14, 21	77
	None	102	8	0	0		0
	None	104	10	0	0		0
	None	107	8	1	1	8	12.5
	None	109	8	3	1	12	12.5
I-17	12	105	10	10	10	2, 2, 2, 3, 3, 3, 3, 3, 4, 4	100
	12	107	4	4	4	2, 2, 3, 4	100
	None	105	10	0	0		0
	None	108	4	0	0		0
D-24	12	103	4	0	0		0
	12	108	4	4	4	6, 6, 8, 10	100
	None	104	4	0	0		0
	None	108	4	0	0		0

*Cortisone treatment was begun three days before infection and continued twelve days or until death.
†Numbers refer to estimations of streptococci in inoculum based on colony counts.
‡Numbers refer to rabbits with positive blood cultures at any time during observation.
§Number of days after infection when death occurred, in individual rabbits.

Todd-Hewitt broth containing varying numbers of one of the four different strains of group A hemolytic streptococci. Blood cultures were made in all animals on the day after infection, and thereafter at intervals of two or three days.

Cortisone, in a dose of 12 mg. each day, was injected intramuscularly into seventy-six rabbits. On the third day each rabbit was infected with one of the strains of streptococci. The daily injections of cortisone were continued until the death of the animal, or for a maximum of twelve days.

A control group of eighty-six untreated rabbits was infected at the same time as the cortisone-treated animals, employing the same strains of streptococci in similar and larger doses.

The results are summarized in Table I, in which it will be seen that seventy-two of the cortisone-treated rabbits developed positive blood cultures for streptococci, and that seventy-one died within two weeks after infection, while eighty of the untreated rabbits did not develop bacteremia and eighty-three survived. Three of the strains appeared to be similar in their pathogenicity for cortisone-treated animals, although titrations to determine end points of virulence were not performed. The fourth strain, D-24, seemed to be less infective under these conditions, since 10^4 streptococci failed to cause septicemia or death. Further studies on the possible application of this method to compare the virulence of different strains are in progress.

The capacity of a smaller dose of cortisone to bring about fatal streptococcal infection was demonstrated in a group of nine rabbits. These animals were given 6 mg. daily, and infected with strain AS0-3 on the fourth day. The results are included in Table I. Eight of the animals developed bacteremia and seven died, while in a control group of ten untreated animals the same inoculum of streptococci caused neither bacteremia nor death.

The Effect of Cortisone Treatment Without Streptococcal Infection.—During the month of March, 1951, four rabbits which were receiving daily injections of cortisone in preparation for streptococcal infection became ill and developed severe diarrhea on the third day of treatment. Each of these animals was found to have septicemia due to *Salmonella typhimurium*. Death occurred within forty-eight hours. No other instances of diarrhea or septicemia were observed in the animal quarters, but it was suspected that latent salmonella infection in the stock might have activated by cortisone.

Except for these four animals, no instances of spontaneous bacterial infection were demonstrable in rabbits undergoing treatment with cortisone, whether or not streptococcal infection was induced. A group of forty rabbits was given 12 mg. of cortisone daily for eight days in order to test the possibility that cortisone alone might have accounted for some of the deaths in the infected animals. All these rabbits survived without apparent ill effect, and none developed blood stream infections.

The Time of Occurrence of Bacteremia and Death.—In approximately 75 per cent of the rabbits which developed streptococcal bacteremia, the blood cultures were positive twenty-four hours after infection, and all were positive within seventy-two hours. Colony counts showed less than 100 streptococci

per milliliter of blood in the first cultures and steadily increased until the day of death, at which time counts as high as 100,000 were occasionally recorded. It was of interest that the animals displayed little evidence of illness during the first three or four days after infection, despite extensive bacteremia. They remained alert and active, and took food and water in the same amounts as control rabbits. When death occurred between the second and fourth days it was extremely sudden, often preceded by a brief series of convulsive sei-

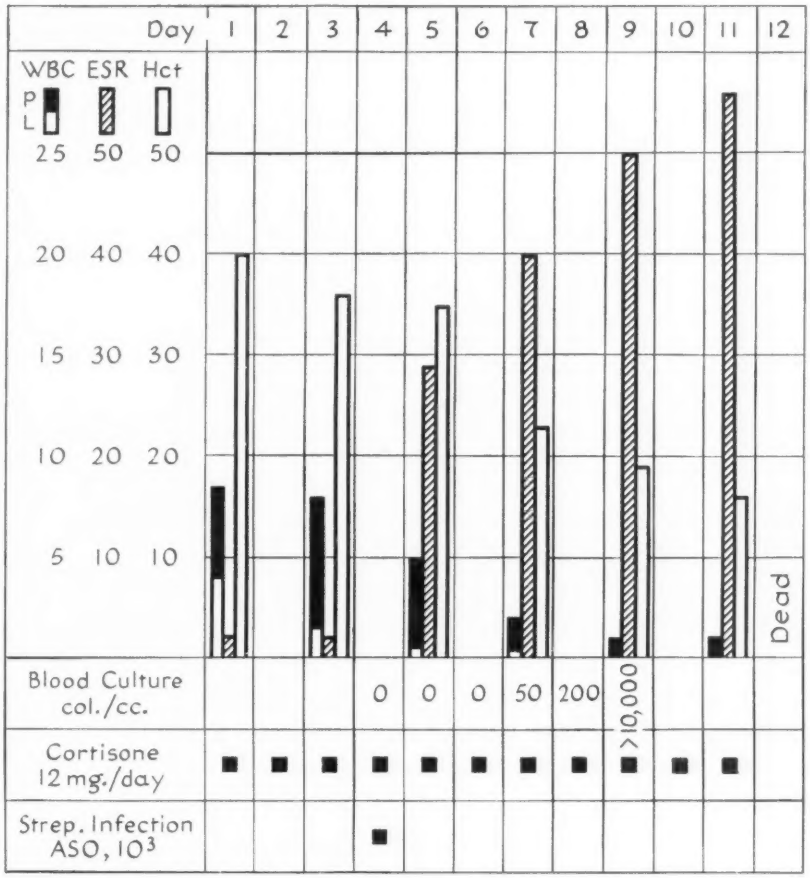

Fig. 1.—Hematologic observations in a cortisone-treated rabbit during infection with group A hemolytic streptococci. *Wbc,* leukocytes ×1000 per cubic millimeter blood; *P,* polymorphonuclear cells; *L,* lymphocytes; *ESR,* erythrocyte sedimentation rate, mm. per hour; *Hct,* hematocrit, per cent packed cells.

zures. Rabbits which survived for a week or longer displayed gradually increasing weakness, loss of appetite, and weight loss, but even these animals were not incapacitated until shortly before death.

The comparatively low colony counts in the first positive blood cultures indicated that the development of systemic infection might be a gradual process, rather than an immediate showering of the blood stream with streptococci from the infected skin site. This was further substantiated by the ob-

servation, in a group of ten cortisone-treated rabbits, that blood cultures taken eight hours after infection were sterile, while all were positive at twenty-four hours.

The time at which death occurred is indicated in Table I. It will be seen that the majority of rabbits died before the tenth day; a few survived for longer periods, and one remained alive for twenty-one days with continual bacteremia. There was considerable variation in the duration of survival among rabbits receiving identical amounts of cortisone, and no relation to the number of streptococci used for infection could be demonstrated.

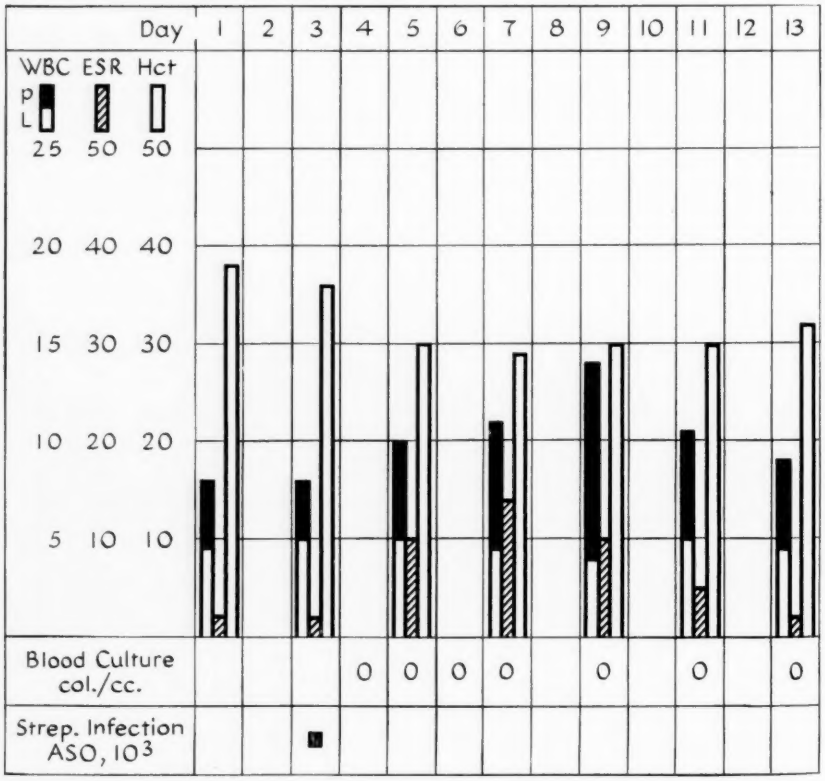

Fig. 2.—Hematologic observations in a rabbit during infection with group A hemolytic streptococci, without cortisone. Symbols as in Fig. 1.

The Effect of Treatment With Penicillin.—Ten rabbits were given cortisone and infected with streptococci by the method described above, and all developed bacteremia. On the third day after infection treatment was started with aqueous penicillin G in a dosage of 100,000 units every six hours. On the following day the blood cultures were negative in all, but 3 died. In the remaining seven rabbits the daily injections of both cortisone and penicillin were continued for four days, and all survived without recurrence of bacteremia. In a comparable group of animals which were not treated with penicillin, bacteremia and death occured in all.

In other experiments it was found that penicillin was not effective when smaller doses were given or when treatment was delayed for longer than three days after infection.

Hematologic Studies.—Total and differential leukocyte counts, sedimentation rate, and hematocrit determinations were made at intervals of one to two days in a group of thirty-five rabbits subjected to cortisone treatment and streptococcal infection. Similar studies were made in comparable groups of animals which received cortisone alone, or streptococcal infection alone. The results of these determinations were remarkably uniform, and are illustrated graphically by examples from each group in Figs. 1 to 3.

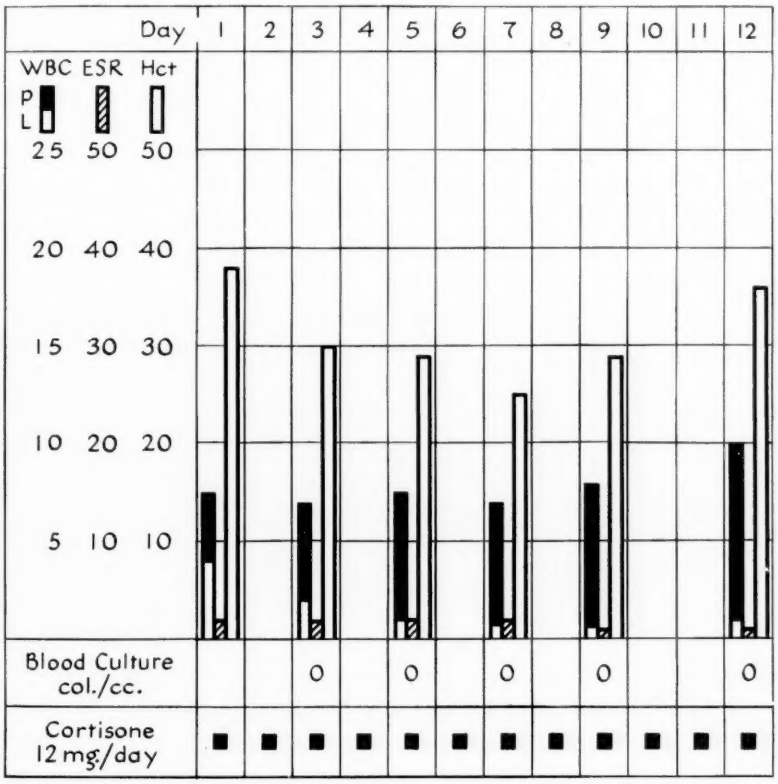

Fig. 3.—Hematologic observations in a cortisone-treated rabbit without streptococcal infection. Symbols as in Fig. 1.

In normal rabbits, the total white cell count ranged from 5,000 to 12,000 and the lymphocyte count from 2,500 to 5,000. The normal sedimentation rate by the Wintrobe method was less than 2 mm. per hour, and the normal hematocrit reading was approximately 40 millimeters.

Within two days after the beginning of treatment with cortisone, the lymphocyte count fell abruptly to a level between 500 and 1,500. When bacteremia became demonstrable in an infected rabbit (Fig. 1) the total leukocyte

count was observed to be decreased, and as the infection became more extensive the leukopenia became more marked. A similar degree of polymorphonuclear leukopenia was observed in three untreated rabbits which were given several intravenous injections of undiluted broth cultures of streptococci with fatal results, and it is considered to be the result of massive infection. It did not occur in cortisone-treated rabbits which were not infected.

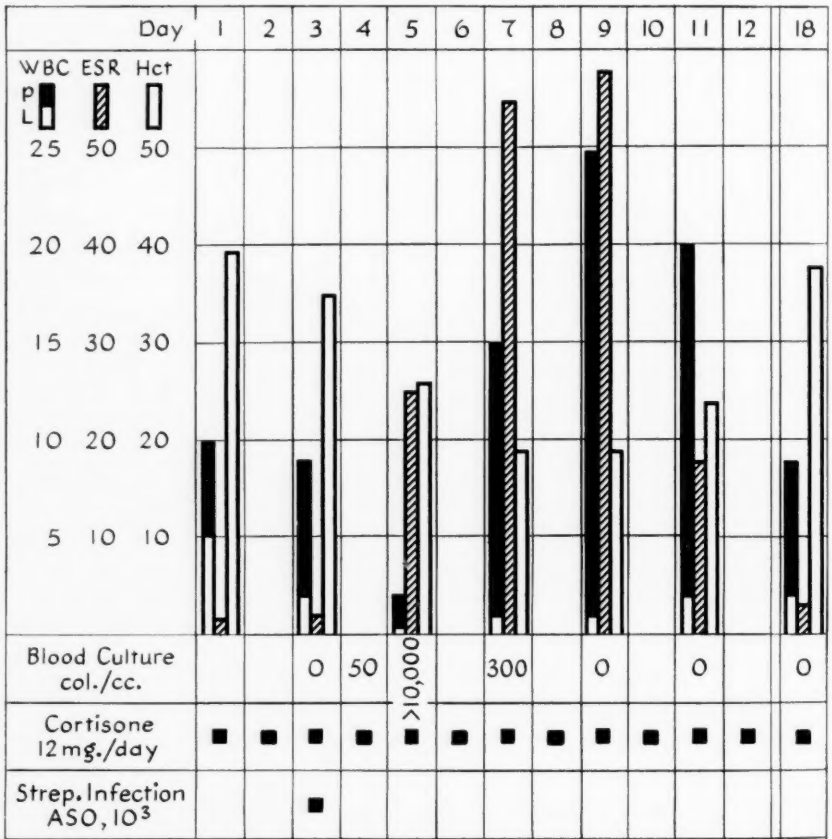

Fig. 4.—Hematologic observations in a cortisone-treated rabbit during infection with group A hemolytic streptococci. Penicillin therapy started three days after infection. Symbols as in Fig. 1.

A mild polymorphonuclear leukocytosis was commonly seen within a day or so after intracutaneous streptococcal infection in the untreated rabbits (Fig. 2). Leukocytosis also occurred in the infected cortisone-treated rabbits when recovery was induced by penicillin (Fig. 4), and the total white counts in such animals reached levels as high as 50,000. In contrast, the lymphocyte count remained low as long as cortisone was administered.

The sedimentation rate became markedly elevated as soon as bacteremia occurred, and usually continued to increase until the time of death (Fig. 1). In the untreated rabbits there was also an increase in the sedimentation rate following infection, but uniformly less than in the animals with bacteremia. The rabbits treated with cortisone alone showed no alteration in the sedimentation rate.

A fall in the hematocrit was observed in all rabbits during the period of observation, and may have been due in part to the frequent bleedings to which they were subjected. However, an extensive fall was only observed in the animals with bacteremia, in which readings of less than twenty were frequently made. An explanation is not available for this change, although it is probable that some destruction of erythrocytes occurred a a result of streptococcal infection and contributed to the fall in hematocrit. It is noteworthy that hemolysis was not demonstrable by tests for hemoglobin in the plasma or urine of fatally infected rabbits, even when great numbers of streptococci were present in the blood.

The phagocytic activity of leukocytes in the blood from cortisone-treated rabbits was compared with that of normal animals, by the method of Huddleson.[13] No significant difference in phagocytosis was demonstrable. Suspensions of heat-killed streptococci were injected into the pleural space of normal and treated animals, and the fluid withdrawn for examination one and three hours later; the extent of phagocytosis appeared to be the same in both groups. Although these methods did not permit quantitative evaluations of phagocytic activity, the results suggested that impairment of this function was not a probable explanation for the effect of cortisone on streptococcal infection.

The Effect of DCA.—It has been reported by others[14] that some of the effects of cortisone may be inhibited by treatment with desoxycorticosterone acetate (DCA) when the diet contains sufficient sodium chloride. Accordingly, the effect of DCA on streptococcal infection in cortisone-treated rabbits was studied. Twelve animals were given cortisone in a dosage of 12 mg. per day, and at the same time 10 mg. of DCA were injected daily; sodium chloride was added to the drinking water in a concentration of 1 per cent. Streptococcal infection was induced on the third day after beginning treatment, employing a strain known to be lethal for cortisone-treated rabbits. A control group of twelve infected animals received cortisone daily without DCA, or added salt in the diet. The results were entirely negative. All the animals in both groups developed bacteremia and died, and no significant differences in survival time were discernible. By itself, DCA had no enhancing effect on the course of experimental streptococcal infection in rabbits, when given in an amount of 10 mg. daily with 1 per cent salt.

The Effect of Nitrogen Mustard.—In a separate study[15] it has been shown that the administration of nitrogen mustard (methylbis [β-chloroethyl] amine hydrochloride) prevents the appearance of bilateral cortical necrosis of the kidneys in cortisone-treated rabbits after an intravenous injection of endotoxin from certain gram-negative microorganisms. In view of the possibility that

nitrogen mustard might exert its effect by interfering with an action of cortisone, the effect of this substance on streptococcal infection in cortisone-treated rabbits was tested. Six animals were injected with 1.5 mg. of nitrogen mustard per kilogram, and treated with 12 mg. of cortisone daily. On the fourth day after the injection of nitrogen mustard, streptococci were injected intracutaneously in a dose sufficient to cause bacteremia and death in other cortisone-treated rabbits. The results showed no evidence of inhibition of the cortisone effect. All of the nitrogen mustard-treated animals died with extensive septicemia within two to three days after infection.

II. The Effect of ACTH on Streptococcal Infection in Rabbits.—The administration of ACTH had an enhancing effect on streptococcal infection which was comparable to that observed with cortisone. However, it was necessary to give large doses of the hormone at intervals of six hours in order to elicit this effect.

Six rabbits were given intramuscular injections of ACTH in a dose of 5 mg. every six hours for seven days, and six other animals were given 10 mg. every six hours. On the fourth day, all twelve rabbits were infected intracutaneously with 10^6 streptococci (ASO-3) and at the same time six untreated rabbits were similarly infected. Blood cultures were made each day after infection.

TABLE II. INCIDENCE OF BACTEREMIA AND DEATH FOLLOWING CUTANEOUS INJECTION OF GROUP A HEMOLYTIC STREPTOCOCCI (STRAIN ASO-3) IN ACTH-TREATED AND UNTREATED RABBITS

ACTH TREATMENT* (MG./DAY)	NO. OF RABBITS	NO. WITH BACTEREMIA	NO. DEAD	MORTALITY (PER CENT)
20	6	2	0	0
40	6	6	5	83
None	6	0	0	0

*Acth injected intramuscularly in equal divided doses, four times daily.

The results are shown in Table II. In the group treated with 20 mg. ACTH daily, bacteremia occurred in two rabbits, but none died. In contrast, all of six rabbits which received 40 mg. ACTH daily developed bacteremia and five died within eight days after infection. None of the untreated rabbits had positive blood cultures and all survived.

III. The Effect of Immunity on Infection in Cortisone-Treated Rabbits.—The capacity of previously immunized rabbits to resist the lethal effect of a streptococcal infection while receiving cortisone was tested in eleven groups of rabbits. Immunization was performed by giving three or more intracutaneous injections of living streptococci in a dose of approximately 10^6 microorganisms, at weekly intervals. One month after the last injection, daily treatment with 12 mg. of cortisone was begun, and on the third day a challenging infection was produced with a suspension of streptococci capable of causing fatal sepsis in cortisone-treated nonimmunized rabbits. As in the earlier experiments described above, cortisone was continued for twelve days. In five of these experiments, the challenging strain was one which had been employed for im-

TABLE III. EFFECT OF PREVIOUS IMMUNIZATION WITH SEROLOGICALLY HOMOLOGOUS AND HETEROLOGOUS TYPES OF STREPTOCOCCI ON THE INCIDENCE OF BACTEREMIA AND DEATH IN CORTISONE-TREATED RABBITS

GROUP	EXPERIMENT NO.	TYPES OF STREPTOCOCCI USED FOR IMMUNIZATION	TYPES USED FOR INFECTION WITH CORTISONE RX	NO. OF STREPTOCOCCI USED FOR INFECTION	NO. OF RABBITS	NO. WITH BACTEREMIA	NO. DEAD	MORTALITY (PER CENT)
Immunized with homologous type	1	1, 3, 17, 24	1	130	10	0	0	0
	2	1, 3, 17, 24	3	10^4	10	1	1	10
	3	1	1	10^6	10	2	1	10
	4	17	17	10^6	10	1	1	10
	5	1	1	10^8	10	10	10	100
Immunized with heterologous type	6	1, 3, 24	17	10^6	5	0	0	0
	7	1, 3, 24	17	10^6	5	0	0	0
	8	1, 3, 24	17	10^8	14	7	7	50
	9	17	1	10^6	10	10	10	100
	10	17	1	10^8	10	10	10	100
	11	1	17	10^6	10	10	10	100
Not immunized	1		1	130	10	10	10	100
	2		3	10^4	10	10	10	100
	3		1	10^6	10	10	10	100
	4		17	10^6	10	10	9	90
	5		1	10^8	10	10	10	100
	6		17	10^6	5	5	4	80
	7		17	10^6	5	5	5	100
	8		17	10^8	10	10	10	100
	9		1	10^6	10	10	10	100
	10		1	10^8	10	10	10	100
	11		17	10^6	10	10	10	100

munization. In the other six experiments, immunized rabbits were challenged with streptococci which were serologically unrelated to the immunizing strains. The results are summarized in Table III.

The first four experiments, involving homologous immunizing and challenging strains, provided definite evidence that immunized rabbits were resistant to the lethal effect of infection combined with cortisone treatment. In each instance, doses of streptococci which were sufficient to cause bacteremia in all the control cortisone-treated rabbits, and death in all except one, were lethal for 10 per cent or less of the immunized animals. However, when a larger dose of streptococci was used for challenge, it was shown that the influence of immunization could be overcome. This is illustrated in Experiment 5, in which 10^8 streptococci caused death in all of ten immunized rabbits. An even larger inoculation of the same strain caused death in only one of twelve control rabbits which were not treated with cortisone (Table I).

In two experiments (Table III, Experiments 6 and 7), immunization apparently produced a high degree of resistance to a heterologous type of streptococcus. In each case, types 1, 3, and 24 were used for immunization, and type 17 (10^6 microorganisms) was given as the challenging infection during cortisone treatment. A larger dose of streptococci (10^8) caused death in 50 per cent of the immunized rabbits, but this dose was fatal for all the controls (Experiment 8).

In the last three experiments, a single strain was employed in three immunizing injections, and the challenge was made with a heterologous strain. No cross-protection was demonstrable between types 1 and 17 by this method.

The data indicate that the enhancement of streptococcal infection by cortisone may be partly counteracted by induced immunity, and that this immunity need not necessarily be type specific. Further studies are necessary to determine whether quantitative differences exist between the immunity produced by homologous and heterologous types of streptococci, and whether the degree of immunity may be related to demonstrable circulating antibody. In regard to the latter point, the titer of antibody against streptolysin 0 did not appear to be related to immunity, as is shown in the section which follows.

The Production of Antibody in Cortisone-Treated Rabbits.—Twelve rabbits were immunized against a type 3 strain of streptococcus by three injections of living microorganisms, and held for six weeks after the last injection. Antistreptolysin 0 titers became elevated in the majority of this group during a period of two or three weeks after starting immunization, but at the end of six weeks the antibody levels were within normal limits (below 1 to 50) in all animals. At this time a course of cortisone treatment, 12 mg. daily, was begun in six of the rabbits. After three days of cortisone, all twelve rabbits were injected intracutaneously with 10^6 streptococci of the same strain used for immunization. Antistreptolysin 0 titers were determined at frequent intervals during a two-week period after this infection.

The results are shown in Fig. 5. It will be seen that antibody titers above the range of normal appeared in four of the cortisone-treated and in five of the untreated rabbits, during the second week after infection. No significant

differences were demonstrable, in the time of appearance or the titer of antistreptolysin 0, between the cortisone-treated and untreated rabbits. It is evident that under the conditions of this experiment no interference with antibody formation by cortisone was demonstrable.

It is noteworthy that all the immunized rabbits in this experiment survived the challenging infection, although significant levels of antistreptolysin 0 were not present in any of their sera at the time of challenge. Moreover, no early rise in antibody was detectable after cortisone treatment was begun, such as might have been expected if cortisone had caused an anamnestic antibody response.

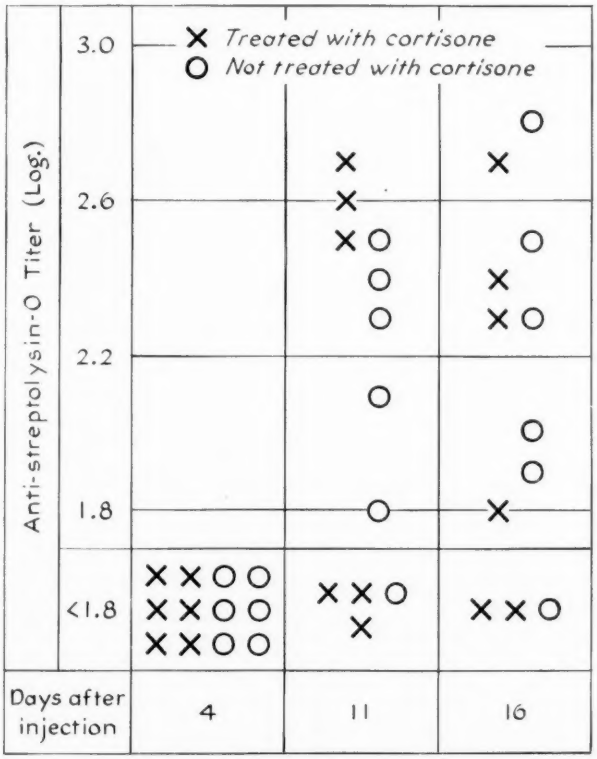

Fig. 5.—Antistreptolysin 0 titers following infection by group A hemolytic streptococci in immunized rabbits, with and without cortisone.

IV. Pathology in Cortisone-Treated Rabbits With Bacteremia.—A striking difference was consistently noted in the local skin lesions at the site of injection in the cortisone-treated and control rabbits. Within two or three days after the infection, the untreated animals exhibited red, rounded swellings of the skin, 3 to 4 cm. in diameter, which subsequently subsided or occasionally formed subcutaneous abscesses followed by ulceration. In contrast, the cortisone-treated rabbits showed no visible reaction at the injected site during the first two or three days, and then frequently developed flat, yellow areas

of dermal necrosis with little gross evidence of inflammation in the surrounding tissues. Several days after infection in some of the treated rabbits extensive intracutaneous hemorrhages appeared at the site of injection, and gradually spread to involve a large portion of the skin of the trunk. These hemorrhages were seen only in animals with extensive, fatal septicemia. Comparable lesions did not occur in the untreated rabbits.

Complete autopsies were performed on twenty-five rabbits which died at various intervals after infection. The most notable gross observations consisted of abundant collections of pink, thin fluid in the peritoneal and pleural spaces, and, in about 60 per cent of the animals, extensive pulmonary hemorrhages. The adrenal glands, spleen, and lymph nodes were smaller than normal in all cases. No gross abnormalities of the heart were noted. The kidneys of several rabbits were moderately enlarged and showed numerous small petechiae over the surface; these lesions did not resemble the extensive cortical necrosis and hemorrhage described elsewhere[2] in cortisone-treated rabbits given intravenous injections of bacterial toxin.

Microscopic sections of the heart in animals which died or were sacrificed within two to four days after infection showed extensive collections of cocci in the parenchymal tissue, grouped in masses in the perivascular spaces and between the muscle fibers. An illustrative photograph of such deposits is shown in Plate 1, *1*; this tissue was placed in fixative within a few minutes after death of the animal, excluding the possibility of post-mortem growth. It is of interest that little or no inflammatory cellular reaction to these microorganisms was observed in rabbits dying at this stage of infection. In the rabbits which died seven days or longer after infection, accumulations of streptococci in the heart tissue were not observed. In several of these animals many areas of myocardial necrosis were seen, with infiltration by round cells, plasma cells, and cells with the appearance of "owl-eyed" cells. An illustrative lesion of this type is shown in Plate 1, *2*. Most of the inflammatory lesions appeared to be located in the immediate vicinity of small blood vessels. The valves showed no abnormality except for an increased number of round cells in the valve leaflets in a few rabbits.

Further studies of the pathology of the heart in rabbits which survived infection during administration of cortisone, and in immunized animals challenged with streptococci while receiving cortisone, are in progress and will be reported in a later communication.

In kidney, liver, spleen, and other tissues, small groups of cocci were frequently seen inside the blood vessels, but deposition of microorganisms in the extravascular tissues was rarely demonstrable, and never in such dense accumulations as in the heart. The liver cells were large and foamy, with an appearance consistent with increased glycogen deposition. The adrenals showed marked generalized thinning of the cortex. The spleen appeared to contain fewer lymphoid cells than normal. The brain was normal except for occasional cocci in the vessels and perivascular spaces. The lungs showed varying degrees of interstitial pneumonitis, with occasional areas of hemorrhage in the alveoli.

DISCUSSION

It has been shown that the administration of cortisone to rabbits produces a profound alteration in the relationship between host and parasite which is overwhelmingly in favor of the parasite. The normal rabbit is highly resistant to the streptococcus; intracutaneously injected microorganisms cause a vigorous local inflammatory reaction and sometimes considerable local tissue damage, but the extension and systemic spread of the infection are effectively

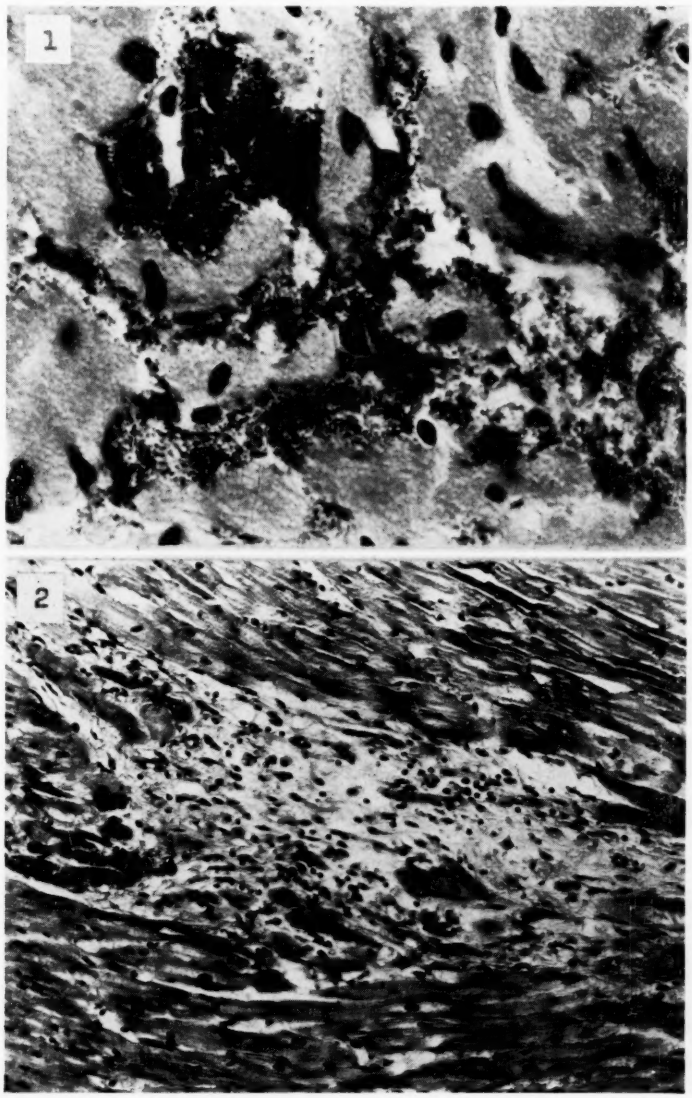

Plate 1.—*1*, Myocardium of a cortisone-treated rabbit infected with group A hemolytic streptococci by the intracutaneous route. This animal died 3 days after infection. Note the accumulation of cocci between muscle fibers. *2*, Myocardium of a cortisone-treated, infected rabbit which died fourteen days after infection. Cocci were not visualized in heart tissue. This section shows an area of muscle fiber necrosis with infiltration by round cells.

prevented. In the cortisone-treated animal the local response in the skin is either lacking or delayed and ineffectual, and there appears to be no restraint of the growth of bacteria when they have reached the circulating blood. The extent of infection, as measured by colony counts of the blood or by histologic examination of the tissues, leads to the impression that the animal participates in the infectious process more as a culture medium than an invaded host.

In order to account for the experimental results, an explanation is needed for two events: the failure of the host to localize the infection at the injected skin site, and the failure to deal with the microorganisms after their appearance in the circulating blood. The latter point is of importance. It is well known that lethal streptococcal infections may be produced in rabbits more readily by intravenous injection than by the intracutaneous route, but the incidence of sustained bacteremia and death is very much less than in cortisone-treated rabbits given intracutaneous infections. In a separate investigation now in progress, massive inocula of the streptococcal strains used in these experiments have been injected intravenously, and bacteremia and death have occurred in less than 10 per cent of the rabbits. It is evident that a mechanism which is available to the normal rabbit for disposing of streptococci in the blood stream is incapacitated in the cortisone-treated animal.

Among the possible explanations for these events, the following may be mentioned. There is substantial evidence that a local inflammatory reaction impedes the spread of infectious agents,[16] and one effect of cortisone is to suppress this reaction. There is reason to believe that lymphoid cells and tissues constitute an important defense mechanism against infectious agents in the blood stream, and cortisone is known to bring about a depletion of lymphoid cells and a reduction in the amount of lymphoid tissues.[17] In this respect, the reaction to streptococci in the cortisone-treated rabbit may be analogous to the lethal sepsis which has been demonstrated in x-radiated animals.[18] The reported capacity of cortisone to decrease capillary and membrane permeability[19] may be a factor in protecting microorganisms in the tissues from cellular or protein constituents of the blood with defensive properties.

It does not seem likely that the polymorphonuclear leukopenia observed in rabbits with bacteremia was primarily involved in the enhancement of infection, although it may have contributed to the lethal outcome as a secondary factor. Such leukopenia did not occur until the streptococci were well established in the blood; moreover, it has been observed in occasional untreated rabbits which were subjected to massive streptococcal infection.

Two factors which influence the infection in favor of the cortisone-treated host were encountered. Penicillin, when administered in large doses within three days after injection, brought about recovery in seven of ten animals. The observation that penicillin was effective when daily cortisone treatment was continued suggests that cortisone does not interfere with the action of this antibiotic, but more information on this point is needed in view of the fact that extremely large quantities of penicillin were employed.

The second factor was immunity. When rabbits were immunized beforehand, by repeated injections of living streptococci, they became considerably

more resistant to the enhancing effect of cortisone on a new streptococcal infection. This observation has several interesting implications. It is apparent that cortisone acts upon "natural" defense mechanisms which are distinct from those which are responsible for active immunity; the latter probably include, in the case of streptococcal infection, phagocytosis.[20] In the present study no impairment of phagocytosis by cells from cortisone-treated animals was demonstrable, although the validity of the methods for studying this function is open to question. The factors involved in natural resistance to streptococcal infection are unknown, and it is possible that further study of the effects of cortisone may shed light on this important problem.

It is of considerable interest that rabbits which had been immunized with several strains of streptococci were resistant when challenged with heterologous serological types during cortisone treatment. There is evidence in the work of others that M protein, which is responsible for type-specificity, plays an important role in streptococcal immunity.[21, 22] The present observations suggest that there may be other antigenic components of streptococci which are involved in the production of immunity. Further studies on this problem are in progress.

No impairment of the formation of antibody during treatment with cortisone was demonstrable when immunized rabbits were challenged with a new streptococcal infection. In six cortisone-treated animals the increase in titer of antistreptolysin O was essentially the same as that in six untreated rabbits. This finding merely adds to the series of conflicting reports concerning the effects of cortisone on antibodies,[23, 24] and is without real significance in the absence of information concerning the identity of the antibody which mediates resistance in streptococcal immunity.

Desoxycorticosterone, with added salt in the diet, did not interfere with the enhancement of streptococcal infection by cortisone, although it has been demonstrated that an antagonism between DCA and cortisone exists in other biological functions.[14] No explanation is available except that the various properties of cortisone may involve separate modes of action.

The failure to inhibit infection by nitrogen mustard in cortisone-treated animals was an expected finding. Although this substance blocks the renal necrosis produced by bacterial toxins in cortisone-treated rabbits, it is more likely that it affects the action of toxin than that of cortisone.[15]

A surprising feature of these experiments was the apparent well-being of infected rabbits during cortisone treatment as judged by their activity and ability to take food in spite of extremely extensive bacteremia. In some instances animals displayed no evidence of illness for several days although blood cultures revealed more than 1,000 streptococci per cubic centimeters during this time. These animals did not die until the microorganisms had multiplied to the extent that colony-like accumulations of cocci were visible within blood vessels and tissues in histologic preparations.

The striking depositions of streptococci in the myocardial parenchymal tissue are of considerable interest. Similar bacterial accumulations in the heart have not been observed in this laboratory in streptococcal infections

without cortisone. It is of added interest that other tissues in the cortisone-treated animals contained fewer demonstrable cocci, and when present they were mainly within the blood vessels. The possibility that streptococci may have an affinity for the heart under these conditions warrants further consideration. It is possible that the areas of myocardial necrosis and inflammation which were encountered in the hearts of rabbits dying after prolonged infections, illustrated by the example in Fig. 2, were the result of tissue damage by streptococcal invasion during the early stages of the infection.

The administration of ACTH produced enhancement of infection which was comparable to that observed with cortisone. Relatively large doses at frequent intervals were required. The sensitivity of rabbits to the effects of ACTH and cortisone may differ, and conclusions regarding the relative capacity of the two substances to produce bacteremia are not warranted at the present time.

SUMMARY

Streptococcal bacteremia and death occurred in seventy-one of seventy-six rabbits which were treated with cortisone in a dose of 6 mg. per kilogram before and after an intracutaneous infection with group A streptococci. Seven of nine rabbits which received 3 mg. per kilogram of cortisone developed bacteremia and died. Of eighty-six infected control rabbits which did not receive cortisone, six developed bacteremia, and three died.

The existence of severe bacteremia did not appear to affect the general appearance, activity, or appetite in many of the cortisone-treated rabbits until shortly before death.

Hematologic studies showed a fall in the lymphocyte count during treatment with cortisone. After bacteremia became demonstrable, there was a fall in the polymorphonuclear leukocyte count, elevation of the sedimentation rate, and marked lowering of the hematocrit. No difference in the phagocytic activity of leukocytes from cortisone-treated and normal rabbits was demonstrable.

The effect of cortisone on streptococcal infection was not modified by the administration of DCA and salt, nor by nitrogen mustard.

The administration of penicillin caused recovery of the majority of cortisone-treated rabbits when given in large doses within three days after infection.

ACTH, in a dose of 5 mg. per kilo every six hours, produced streptococcal bacteremia and death in five of six infected rabbits.

Immunization of rabbits with living streptococci resulted in resistance to the enhancing effect of cortisone on streptococcal infection, with either homologous or heterologous serological types. The formation of antistreptolysin O occurred at the same time and in approximately the same titer in cortisone-treated as in normal rabbits.

Large accumulations of cocci were visible in histologic preparations of tissues from cortisone-treated rabbits which died within two to four days after infection. Deposits of cocci were most prominent in the myocardial

parenchyma, and within the blood vessels in other tissues. In rabbits which died after seven days cocci were not observed in the tissues, and foci of myocardial necrosis and inflammation were present in the heart.

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SENSITIVITY STUDIES ON HUMAN PLEUROPNEUMONIA-LIKE ORGANISMS

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THE determination of the susceptibility of pleuropneumonia-like organisms to antibiotics, Myochrysine, and nitrogen mustard was undertaken as part of a project on the study of collagen diseases. The clinical observations of this same study and their correlation with laboratory data have been published elsewhere.[1-3]

In recent years interest in this group of organisms has been manifested and stimulated by many important contributions, admirably summarized and interpreted by Klieneberger-Nobel in her paper on filtrable forms of bacteria.[4] Since their discovery the interest in these organisms has centered essentially around three spheres: (1) their role in animal diseases, (2) their potential pathogenicity for man, and (3) the fundamental importance of the L form as a phase of bacteria.

The cultivation of the etiologic agent of bovine pleuropneumonia[5] and the demonstration that agalactia of sheep and goats is caused by a similar organism[6-8] established the role of pleuropneumonia organisms as animal pathogens. The organisms of pleuropneumonia of cattle and of agalactia are the prototypes of the group of pleuropneumonia and pleuropneumonia-like (L) organisms. Klieneberger[9] refers to this group as a distinct family, whose members resemble each other closely, but differ widely from other known microbes. Their morphologic characteristics have been studied in detail by many authors[9-12] and "inclusion of a microorganism in the pleuropneumonia group is based on the morphologic similarity of the colonies to the organism producing contagious pleuropneumonia bovis.[13]" (Fig. 1.)

It is of interest that several of the animal strains of L organisms such as the L_4 organism, the M55 strain, and Sabin's mouse strains, Types B, C, and D, as well as the organism of agalactia have a definite affinity for joints.[9, 16]

The relationship of pleuropneumonia-like organisms to human disease and, in particular to diseases of the joints and genitourinary tract, is controversial. Their potential pathogenicity, however, is suggested by many observations. Streptobacillus moniliformis with its L phase is pathogenic for human beings as well as for animals.[17] Its affinity for joints in man, animals and the chick embryo has been clearly demonstrated.[17, 18] This organism, therefore, assumes

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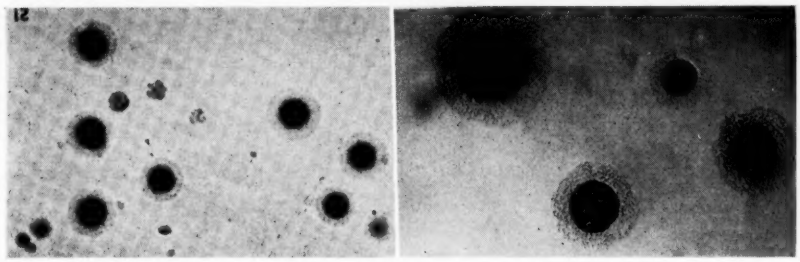
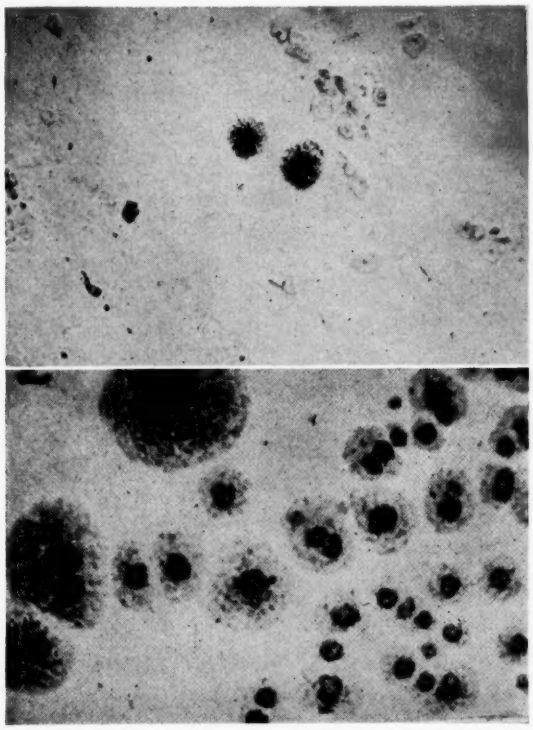
special importance because L organisms, isolated as independent strains from human or animal sources, seem highly species specific.[13, 19] This apparent species specificity has made it impossible thus far to produce disease in experimental



A. *B.*

Fig. 1.—*A*, Colonies of pleuropneumonia (strain Shanghai). Reproduced from Ledingham (Ref. 14). (×70.) *B*, colonies of the L_1 strain separated from *Streptobacillus moniliformis* (Ref. 17). (×70.)



C.

D.

Fig. 1.—*C*, Culture of pleuropneumonia-like organisms, isolated from cervix. (×70.) *D*, 3A L type colonies of *Salmonella typhimurium*. Reproduced from Weinberger, Madoff, and Dubnes (Ref. 15). (×30.)

animals with human strains, and the assessment of the role of L organisms in human disease must at present depend on observations in patients combined with bacteriologic studies.

The cultivation of human strains of pleuropneumonia-like organisms from apparently normal tissues[20-22] has made it difficult to evaluate their significance in the pathogenesis of various diseases with which they may be associated. Perhaps one should regard them as "opportunist pathogens," organisms often saprophytic in human tissues, of relatively low virulence, but gaining pathogenic importance when given the opportunity, e.g., injury, lowered resistance of the host, massive infection, etc. Such potential pathogenicity for man is suggested by the reports of the finding of pleuropneumonia-like organisms in pure culture in a suppurating Bartholin's gland,[23] cases of cystitis and abacterial pyuria,[24, 25] a tubo-ovarian abscess,[21] and the spinal fluid of a patient with meningitis and brain abscess.[13]

The possible relationship of pleuropneumonia-like organisms to human joint disease, genitourinary tract disease, and ulcerative colitis has been discussed repeatedly.[1, 20, 24, 26-32] Their role as an antigen responsible for the hypersensitivity state in collagen diseases has also been suggested.[2, 3] Pleuropneumonia-like organisms have now been demonstrated repeatedly in joint fluid of patients with Reiter's disease[19, 31, 33] and once in the joint fluid of a case of arthritis following pneumonia.[2]

The suggestive association of L organisms and these diseases led us to study the effect of various compounds on human strains which were derived from patients with rheumatic and genitourinary tract diseases. This report summarizes the results obtained.

EXPERIMENTAL

Material.—Twenty-eight strains of pleuropneumonia-like organisms of human origin and the L_4 strain were used in this study. Fifteen strains were obtained from male patients using prostatic secretion as culture material and thirteen strains were derived from cervical cultures of ten female patients. The diagnoses in these cases included:

Rheumatoid arthritis	9	Arthritis, type undetermined	2
Marie-Strumpell arthritis	2	Gonorrheal arthritis	1
Erythema nodosum	2	Nonspecific urethritis	4
Rheumatic fever	1	Cervicitis (*N. gonorrhoeae*)	1
Chorea	1	Normal	1
Lupus erythematosus	1		

Isolation of Pleuropneumonia-like Organisms.—The medium used for isolation of pleuropneumonia-like organisms was 1.5 per cent pancreatic digest agar[34] pH 7.7, containing 20 per cent of ascitic fluid* or horse serum. A few strains were isolated on 1.5 per cent Bacto-tryptose phosphate agar containing the same enrichment. Agar was sedimented overnight and the sediment discarded. This resulted in a very clear medium through which microscopic colonies could be visualized easily. All cultures were incubated aerobically and anaerobically at 37° C. The aerobic plates were sealed with parafilm to insure proper moisture during prolonged incubation. Duplicate plates containing 25 units of penicillin per milliliter of medium were set up in each instance.

Cultures were examined microscopically (×100) and by stained agar preparations at the end of forty-eight and ninety-six hours, and negative cultures were held for a total of three weeks.

In general more luxuriant growth of L organisms was obtained on pancreatic digest than on tryptose phosphate agar. All strains that grew well aerobically, grew equally well under reduced oxygen tension or under anaerobic conditions. Of a total of seventy-two strains

*Ascitic fluid was heated at 56° C. for one hour.

TABLE I. SENSITIVITY STUDIES ON PLEUROPNEUMONIA-LIKE ORGANISMS MINIMAL LETHAL CONCENTRATION DETERMINED IN LIQUID MEDIA

STRAIN	SEX	MICROGRAMS PER MILLILITER					UNITS PER ML.
		TERRAMYCIN	CHLORAMPHENICOL	AUREOMYCIN	STREPTOMYCIN	SODIUM AUROTHIOMALATE	PENICILLIN
HA	M	4	16	8	128	64	51200
WH	F	4	32	32	512	64	51200
WA p.	M	4	64	16	512	64	51200
GA	F	4	256	64	2048	32	51200
TE	F	8	16	16	1024	16	25600
LO an.	M	8	32	16	128	32	51200
CL	M	8	32	32	512	32	51200
SH p. an.	M	8	32	128	4096	128	51200
TE p.	F	8	64	64	1024	64	51200
NO	M	8	128	64	256	64	25600
BRE	M	16	32	16	1024	64	25600
YE an.	M	16	32	128	512	128	51200
KE	F	16	64	16	1024	64	51200
CR	M	16	64	32	2048	32	51200
PO p.	F	16	64	128	512	64	51200
BRA	M	16	128	128	1024	64	51200
GO p.	F	16	128	128	2048	64	51200
PE p.	M	16	256	>256	1024	64	51200
GA p.	F	32	64	64	1024	32	51200
CH p.	F	32	64	64	2048	64	51200
DA	M	32	64	128	512	64	51200
GI p.	M	32	64	256	1024	32	51200
TH an.	F	32	64	256	4096	32	51200
MO	F	32	128	256	2048	32	51200
RY	M	32	512	256	1024	64	51200
CH* p.	M	64	64	>256	512	64	51200
ST	F	64	128	32	512	32	51200
CH	F	256	512	256	512	64	51200
L_4		256	512	>256	2048	2048	51200

p.—Isolated from penicillin medium.
an.—Anaerobic strain
*—Husband of patient CH

we have encountered eleven strains which could be isolated under anaerobic conditions only. Five of those eleven required anaerobiosis for maintenance. Subcultures of the other six strains could be maintained under reduced oxygen tension. Four anaerobic strains were used in this study, and will be designated as such.

Penicillin was used to facilitate isolation of L organisms from the ordinary bacteria. When possible, however, the strains were isolated without the aid of penicillin. The organisms isolated from penicillin media will be designated as P strains. In only one instance have we noted L organisms on penicillin plates that were not demonstrable on penicillin-free media.

Stock cultures were maintained by transfers every twenty-four to forty-eight hours in 20 per cent ascitic fluid Bacto-tryptose phosphate broth or by preservation at −18° C. The frozen broth cultures have remained viable for one year or longer.

Determination of Sensitivity.—The substances studied for their effect on L organisms were aureomycin, chloramphenicol, streptomycin, terramycin, penicillin G, sodium aurothiomalate (Myochrysine) and nitrogen mustard.

Solid and liquid media were used to determine degrees of inhibition as well as the lethal effect of the various compounds used.

The basic liquid medium was 20 per cent ascitic fluid tryptose phosphate broth, to which the various agents were added in serial dilutions as indicated in following charts. 4.9 ml. of this medium were inoculated with 0.1 ml. of an undiluted twenty-four hour broth culture. The tubes were incubated for forty-eight hours at which time the human strains showed no or very faint turbidity. One-tenth milliliter of the broths was then inoculated on 20 per cent ascitic fluid pancreatic digest agar plates. Growth was recorded after forty-eight hours of incubation.

The figures in Table I represent the minimal lethal concentrations of the various agents. Human L organisms are noted for their property of growing abundantly in broth without producing turbidity. Exposure of these organisms to antibiotics incorporated in liquid media is therefore useful only to determine the lethal effect of various compounds, but gives no information on their inhibitory effect.

The newer antibiotics are known to be bacteriostatic rather than bactericidal agents.[35-37]

The antibiotics were therefore incorporated in solid media in order to observe any inhibitory effect. The basic medium was 20 per cent ascitic fluid pancreatic digest agar, to which the different antibiotics were added to give the final concentrations indicated in the charts. This medium was inoculated with 0.1 ml. of a 1-100 dilution of a twenty-four hour broth culture. Partial and complete inhibition were recorded after forty-eight hours of incubation.

TABLE II. COMPARISON OF INHIBITORY AND LETHAL EFFECT

	STRAIN		MICROGRAMS PER MILLILITER		
			MIC	CIC	MLC
Terramycin	HA		0.125	0.25	4
	SH	p. an.	0.125	0.5	8
	GI	p.	0.25	0.5	32
	CH		1	2	256
Chloramphenicol	SH	p. an.	0.5	4	32
	GI	p.	2	8	64
	CH		2	4	512
	L_4			8	512
Aureomycin	SH	p. an.		2	128
	GI	p.	4	16	256
	CH		4	8	256
	L_4		8	16	>256
Streptomycin	SH	p. an.	8	16	4096
	GI	p.		128	1024
	CH		4	8	512
	L_4		16	128	2048
Sodium aurothiomalate	GI	p.		32	32
	HA			32	64
Penicillin			Units Per Milliliter		
	SH	p. an.		51200	51200
	L_4		12800	25600	51200

MIC—Minimal inhibitory concentration.
CIC—Concentration necessary for complete inhibition.
MLC—Minimal lethal concentration.
p.—Isolated from penicillin medium.
an.—Anaerobic strain.

From all plates not showing L colonies, agar blocks measuring approximately 1 sq. cm. were cut out and inoculated into 20 per cent ascitic fluid tryptose phosphate broth. After incubation for forty-eight hours 0.1 ml. of undiluted broth was inoculated onto the standard solid medium containing no antibiotic and growth recorded after forty-eight hours of incubation.

TABLE III. RELATION OF LETHAL TO INHIBITORY CONCENTRATIONS

	STRAIN		MICROGRAMS PER ML.		RATIO	NUMBER OF STRAINS WITH
			C I C	M L C	M L C / C I C	SAME RATIO
Terramycin	WH		2	4	2	4
	TE		2	8	4	4
	LO	an.	1	8	8	3
	HA		0.25	4	16	9
	CR		0.5	16	32	5
	GI	p.	0.5	32	64	2
	L_4		1	256	256	2
Chloramphenicol	TE		8	16	2	1
	TH	an.	16	64	4	1
	SH	p. an.	4	32	8	7
	YE	an.	2	32	16	10
	PO	p.	2	64	32	6
	L_4		8	512	64	1
	RY		4	512	128	3
Aureomycin	TE		8	16	2	2
	CR		8	32	4	3
	NO		8	64	8	9
	GI	p.	16	256	16	6
	YE	an.	4	128	32	6
	SH	p. an.	2	128	64	3
Streptomycin	HA		64	128	2	1
	LO	an.	16	128	8	2
	L_4		128	2048	16	3
	NO		8	256	32	4
	CH		8	512	64	9
	KE		8	1024	128	5
	YE	an.	2	512	256	4
	BRE		2	1024	512	1
Penicillin	SH	p. an.	51200†	51200	1	7
	GI	p.	25600	51200	2	22
Sodium aurothiomalate*	GI	p.	32	32	1	16
	HA		32	64	2	3
	SH	p. an.	32	128	4	1
	L_4		128	2048	16	1

CIC—Concentration necessary for Complete Inhibition
MLC—Minimal Lethal Concentration
p.—Isolated from penicillin medium
an.—Anaerobic Strain
*Only 21 of 29 strains reported.
†Units per ml.

TABLE IV. SENSITIVITY STUDIES ON L ORGANISMS (Complete inhibition of growth on solid media)

STRAIN	SEX	MICROGRAMS PER MILLILITER						UNITS PER ML.	DIAGNOSIS
		TERRAMYCIN	CHLORAMPHENICOL	AUREOMYCIN	STREPTOMYCIN	SODIUM AUROTHIOMALATE	NITROGEN MUSTARD	PENICILLIN	
HA	M	0.25	2	2	64	32		25600	Sydenham's Chorea
YE an.	M	0.5	2	4	2	128		51200	Rheum. Arth.
SH p. an.	M	0.5	4	2	16	32		51200	Arth. Type?
CR	M	0.5	4	8	8	32		25600	Non- sp. ureth.
GI p.	M	0.5	8	16	128	32		25600	Rheum. Arth.
PO p.	F	1	2	8	8			25600	Rheum. Arth.
KE	F	1	4	4	8			25600	Rheum. Arth.
LO an.	M	1	4	4	16	32		25600	Non- sp. ureth.
CH* p.	M	1	4	8	8		>128	25600	Normal
RY	M	1	4	8	8	64		51200	Rheum. Arth.
GO p.	F	1	4	8	16	64		25600	G. C. cervicitis
BRA	M	1	4	8	32	64		25600	Arth. Type?
GA p.	F	1	4	16	16	32		25600	Rheum. Arth.
CH p.	F	1	4	16	128	64		25600	Eryth. nod.
NO	M	1	8	8	8	64	>128	25600	Rheum. spond.
GA	F	1	8	8	16	32		25600	Rheum. Arth.
PE p.	M	1	8	16	16	64		51200	Non- sp. ureth.
L_4		1	8	16	128	128		25600	
DA	M	2	2	4	4			25600	Rheum. Arth.
CH	F	2	4	8	8	64	128	25600	Eryth. nod.
WH	F	2	4	8	16		128	25600	G. C. Arthr. cervicitis
ST	F	2	4	16	8	32	>128	25600	Rheum. Arth.
TE	F	2	8	8	32			12800	Lupus eryth.
MO	F	2	16	8	32	32		51200	Eryth. nod.
TH an.	F	2	16	16	16	16		25600	Rheum. Fever
BRE	M	4	2	2	2			12800	Rheum. spond.
WA p.	M	4	4	2	8			51200	Non- sp. ureth.
CL	M	4	4	8	32	32	128	25600	Rheum. Arth.
TE p.	F	4	8	16	16	32		25600	Lupus eryth.

By this method it was possible to determine for the given compounds:

1. MIC—Minimal inhibitory concentration. Reduction of number of organisms by 50 per cent or more.

2. CIC—Complete inhibitory concentration. No visible growth on the antibiotic agar.

3. MLC—Minimal lethal concentration. No growth upon transfer to antibiotic-free medium.

As a rule much higher concentrations of the antibiotics were necessary to produce a lethal effect than were required for partial and complete inhibition. This parallels closely the observations made on their bacteriostatic and bactericidal effects on common bacteria.[35] It was found that 2 to 512 times more of the antibiotics was needed for lethal effect than for complete inhibition when terramycin, chloramphenicol, aureomycin and streptomycin were employed. The ratio of lethal to inhibitory concentrations for penicillin never varied by more than two (one tube dilution). A similar effect was observed with Myochrysine in all but two strains.

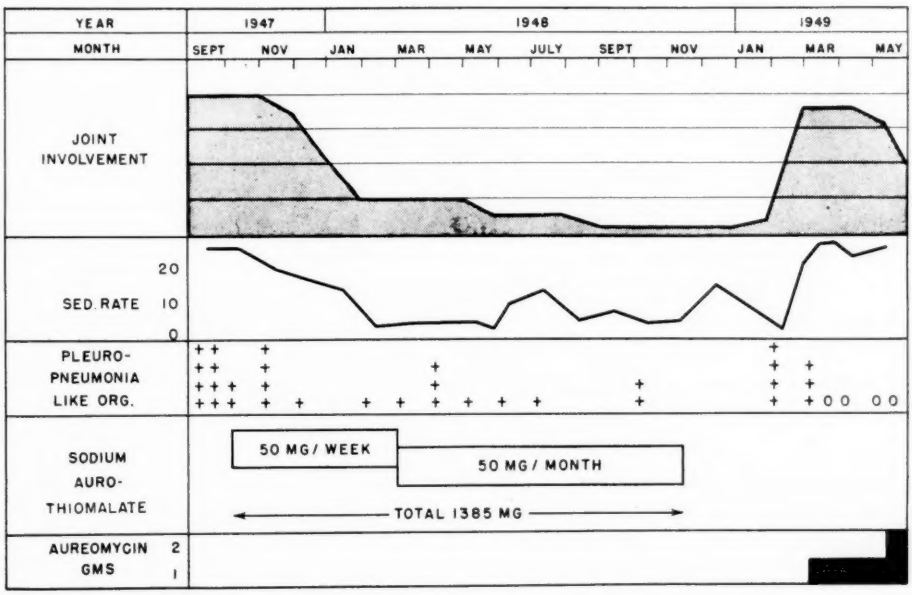


Fig. 2.—Rheumatoid arthritis treated with gold salts and aureomycin. Cultural studies.

Table IV gives the sensitivity of twenty-nine strains of pleuropneumonia-like organisms determined as complete inhibition of growth on solid media. It shows that under the conditions of this experiment, the majority of the strains was more sensitive to terramycin than to any of the other compounds tested. The same relationship could be demonstrated when minimal inhibitory concentration, complete inhibition and lethal effect were studied.

Sodium aurothiomalate (Myochrysine) was included in this study because of its known efficacy in the treatment of rheumatoid arthritis, its effect in experimental arthritis,[38, 39] and its in vitro effect on animal strains of L organisms.[40]

Whereas pleuropneumonia-like organisms persist in vivo, under gold therapy sufficient to induce clinical remission of rheumatoid arthritis,[1] it has been possible to eliminate L organisms in vivo with aureomycin,[1, 41, 42] chloramphenicol, and terramycin in all cases studied. Most of the patients received antibiotics in doses varying between 1 and 2 Gm.

daily, but it has been possible to demonstrate the disappearance of L organisms on small doses of the antibiotics, such as aureomycin, 250 mg. daily, and terramycin, 10 mg. every third day.

In vitro studies with Myochrysine did not produce clear-cut results. In many of the strains a zone phenomenon was observed. Minimal amounts of Myochrysine would inhibit these strains whereas higher concentrations permitted growth, as illustrated in Table V. A similar, as yet unexplained, zone phenomenon has been noted previously in studies on the action of gold salts on the L_7 organism[40] and on *Mycobacterium tuberculosis*.[43]

Nitrogen mustard was inhibitory for pleuropneumonia-like organisms only in high concentrations. Because of this and the exacting precautions necessary, these experiments were not extended.

TABLE V. ZONE PHENOMENON OBSERVED IN SENSITIVITY STUDIES OF L ORGANISMS

STRAIN	MICROGRAMS OF MYOCHRYSINE PER MILLILITER										
	128	64	32	16	8	4	2	1	0.5	0.25	0
CH p.	0	0	+	++	++	0	0	0	0	0	++++
GO p.	0	0	++	++++	+	0	+++	0	0	0	++++
BRA	0	0	+++	++++	++++	+++	+++	+	+	+	++++
PE p.	0	0	20 col.	20 col.	10 col.	0	0	0	0	10 col.	++++
GA	0	0	0	+++	++++	+++	++	+	+	+	+++

p.—Isolated from penicillin medium.

DISCUSSION

A review of the literature shows general agreement as to the effect of gold salts, penicillin, and sulfonamides on pleuropneumonia-like organisms. Various studies published on the effect of streptomycin, aureomycin and chloramphenicol have given conflicting results.

Gold compounds inhibit some strains of pleuropneumonia-like organisms in vitro.[40] They are effective in arthritis, experimentally produced with L organisms.[19, 39, 40] They are accepted as effective clinically in the treatment of rheumatoid arthritis. Myochrysine, in doses therapeutic for rheumatoid arthritis, does not eliminate the L organisms from the human genital tract.

Penicillin and sulfonamides are not only ineffective against pleuropneumonia-like organisms in vivo and in vitro,[17, 24, 38, 44] but are used to study the development of L type colonies and large bodies from a variety of bacteria.[15, 45, 46, 47, 48]

Powell and associates[49] reported that streptomycin appeared therapeutically effective against pleuropneumonia infections and resultant polyarthritis in rats. Encouraging clinical improvement in patients with acute arthritis or Reiter's syndrome and disappearance of L organisms in cases of urinary tract infection during treatment with streptomycin have been noted.[24, 33] The in vitro streptomycin sensitivity of some organisms in this group was 20 μg per milliliter.[24] Dienes and co-workers[50] report that the growth of L forms of *S. typhosa* and *S. typhimurium* and of pleuropneumonia-like organisms isolated from human beings and rats was almost completely inhibited by 10 μg per milliliter, a few colonies, however, developed on plates with the highest concentration tested (45 μg per ml.). Five human strains studied by Hatch[51] were inhibited by 25 μg per milliliter. Leberman and associates[52] reported that nine of twelve human

strains were sensitive to concentrations as low as 0.1 μg of streptomycin per milliliter, and noted a striking difference in sensitivity to streptomycin and dihydro-streptomycin. Twenty-three of twenty-eight human strains, studied by us, were inhibited by 8 to 64 μg per milliliter, three strains were sensitive to 2 μg of streptomycin, and two strains required a concentration of 128 μg per milliliter for inhibition.

It appears that there is, in regard to sensitivity, a marked strain difference among human L organisms. Of interest in this respect are some of the data summarized in Table IV of this paper. It will be noted that complete inhibition of growth of strains CH and CH*^{p} was obtained with 8 micrograms of streptomycin per ml. Strain CH^{p} required 128 micrograms of streptomycin for the same effect. Strain CH was isolated from a cervical culture of a patient with erythema nodosum which yielded abundant growth on penicillin-free media. Prostatic culture of the patient's husband showed only few pleuropneumonia-like organisms (Strain CH*^{p}), which could not be isolated without the aid of penicillin, but L organisms were present on the penicillin-free media. Strain CH^{p}, much more resistant to streptomycin, was again isolated from the patient, but after she had received 250 mg. of aureomycin. Penicillin-free plates showed only very few colonies of L organisms. We could not separate them from the bacterial flora. The plates containing penicillin had many colonies of L organisms, and the more resistant strain CH^{p} was derived from them. This finding raises the question whether one individual may carry more than one type of L organism, or whether alteration in streptomycin sensitivity occurred following administration of aureomycin. Our resistance studies are as yet incomplete. In the few strains tested we have found no development of resistance to aureomycin or chloramphenicol for as many as twelve transfers in media containing these antibiotics in subinhibitory concentrations.

While the varying values obtained by a single observer can be explained by a difference in strain sensitivity, the varying results of different observers are perhaps related more to test method than to susceptibility of organisms. Thus far, every investigator in this field uses a different basic medium with different types of enrichment in an effort to provide optimum conditions for growth of the organisms. Accurate comparison of results must therefore await the extension of studies on growth requirements, such as those reported by Smith and Morton.[53] To obtain comparable results it will also become necessary to adopt certain standard procedures such as have been developed for sensitivity studies of the ordinary bacteria.

Antibiotics and chemotherapeutic agents may antagonize the growth and/or survival of microorganisms. Many of the "newer" antibiotics have been found to be bacteriostatic rather than bactericidal and to exert their effect largely on multiplying organisms.[35, 37, 54] The term sensitivity usually denotes concentrations necessary for complete inhibition of growth of actively growing organisms after a specified time of incubation.

The properties of human L organisms eliminate the use of the tube dilution method and estimation of growth by broth turbidity for the determination of inhibition. Surface inoculation of agar containing antibiotics would therefore be the method of choice. While inocula of standard size and age of culture should

assure a certain consistency of results, the deterioration of some of the antibiotics is more difficult to control. This makes it desirable that readings be done after a minimum time of incubation. Again, the characteristics of human L organisms often make accurate reading impossible before forty-eight hours of incubation. In studies with aureomycin, which deteriorates readily in alkaline and enriched culture media and at incubator temperature, values obtained after forty-eight hours' incubation are possibly higher than the true inhibitory concentration of the antibiotic, and further prolongation of incubation will increase the figures obtained. This, in addition to strain difference, may explain discrepancies in sensitivity reported by various authors.

Paine and collaborators[54] report one strain of pleuropneumonia-like organism to be sensitive to 0.25 μg of aureomycin (length of incubation not stated). Dienes and co-workers[50] noted partial inhibition by 10 μg per milliliter and complete inhibition at higher concentration (incubation time not given). Sensitivity of our strains (forty-eight hours' incubation) varied between 2 and 16 μg per milliliter. Hatch[51] found inhibition of most of his strains by 50 μg per milliliter (seven days' incubation). The higher figures of Leberman and associates[52] are in the range of our determination of minimal lethal concentrations. With the L_4 strain, tube dilution as well as agar method can be used to determine sensitivity. With the tube dilution method we obtained values comparable to those reported by Kuzell for aureomycin,[55] but higher concentrations were necessary to demonstrate inhibition on solid media.

The human strains of L organisms examined by us were more resistant to chloramphenicol than the very sensitive L forms of *S. typhosa*, *S. typhimurium*, and the L_1 culture studied by Dienes.[50] We have not observed the enhancement of growth of pleuropneumonia-like organisms in the presence of chloramphenicol and aureomycin, reported by Leberman and collaborators.[52]

Sixty-two per cent of the strains tested were completely inhibited by 1 μg or less of terramycin. The data presented would indicate that under standard experimental conditions in regard to basic medium, inoculum size and incubation time, terramycin was more effective against human L organisms than any of the other agents tested. The greater stability of terramycin might account for the difference noted in the effect of terramycin and aureomycin, although terramycin also shows some loss of activity in vitro at 37° C.[37]

We are indebted to Dr. Kuzell for sending us a culture of the L_4 organism. This strain was completely inhibited by 1 μg of terramycin in vitro. Rat arthritis, produced experimentally with the L_4 strain, has been useful in evaluating various therapeutic agents, despite the fact that this arthritis is no exact counterpart of human rheumatoid arthritis.[19, 39, 55] It is of interest that Kuzell[19] found that terramycin is effective in preventing the rat polyarthritis and suggested that terramycin be given a clinical trial in Reiter's syndrome and early rheumatoid arthritis.

SUMMARY

Twenty eight strains of pleuropneumonia-like organisms of human origin and the L_4 strain have been studied to determine their susceptibility to aureomycin, chloramphenicol, terramycin, penicillin, streptomycin, sodium aurothiomalate, and nitrogen mustard.

The majority of strains was sensitive to aureomycin, chloramphenicol, and terramycin. Terramycin was more effective than aureomycin or chloramphenicol in most instances.

Minimal lethal concentrations of terramycin, chloramphenicol, aureomycin, and streptomycin were much higher than concentrations required for inhibition. This indicates that these antibiotics exert a static rather than a lethal effect on pleuropneumonia-like organisms.

The technical assistance of Miss Frances Paoliello and Mr. Emerick M. Mulich is gratefully acknowledged.

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LABORATORY METHODS

A NEW METHOD FOR MULTIPLE INOCULATION OF EMBRYONATED EGGS, AND EVALUATION OF THE INJECTABILITY OF SEVERAL COMMON SOLVENTS

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THE embryonated egg is frequently used in the study of viruses, rickettsia, bacteria, and fungi. It is also of value in testing various chemical compounds for their inhibitory and cidal effects, and in this connection it is frequently desirable and necessary to administer multiple injections of the compound being evaluated. Several methods have been described for the injection of eggs.[1-4] Most of these involve single injection techniques and all require repeated handling. None of these methods seemed suitable for our purposes since we desired to work with large numbers of eggs, giving daily injections over a seven-day period. After considerable experimentation a technique was devised which proved to be less time consuming, to minimize contamination, and to decrease the embryo mortality from frequent manipulation. This method is described in detail in the following pages. In addition we have evaluated several of the more common solvents as to the amount injectable per day over a seven-day period and the effects of these solvents upon the developing embryo.

METHOD

1. The hub is removed from a 20-gauge one and one-fourth inch needle.

2. A number 1-XXXX regular cork is cut transversely in half with a sharp knife, either half of which may be used. The beveled pointed end of the above shaft of the needle is inserted through the center of the cork so that the tip of the beveled end of the needle extends 5 mm. beyond the surface of the cork (Fig. 1). A wire is then inserted through the needle to remove any piece of cork which may have lodged there.

3. The other end of the needle is inserted into one end of two feet of plastic tubing* and lightly held in place with a rubber band. A one and one-fourth inch 20-gauge needle is inserted into the other end of the tubing and fastened similarly with a rubber band. The two ends are placed together and wrapped in gauze for autoclaving (Fig. 1).

4. A one cubic centimeter tuberculin syringe is used in these experiments.

5. The rolled border of the opened end of a condom is removed, and the condom is so placed and fastened to the barrel of the syringe with a rubber band that the barrel of the syringe is easily manipulated within the lumen of the condom and does not obscure the marking of the syringe (Fig. 2).

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*Clear, 18-gauge Transflex Tubing obtained from Irvington Varnish and Insulator Company, Irvington, N. J.

6. The open end of the syringe is then wrapped in gauze for autoclaving.

7. The above apparatus is then autoclaved at 15 pounds' pressure for twenty minutes.

8. Ten-day-old embryonated eggs were used throughout these experiments. They were incubated at a temperature of 99.5° F., and with a humidity reading on the wet bulb thermometer of 86-88° F. With the use of a dental drill a 1-mm. hole is made through the eggshell over the chorioallantoic cavity.

9. The syringe is filled with the test substance and attached to the tubing with sterile forceps by means of the hubbed needle. The tubing is then filled with the test substance. The syringe is again disengaged and refilled to the one cubic-centimeter mark and the needle is then firmly reattached.

10. The opening in the eggshell is ringed with nail polish and the bottom of the cork is likewise treated, care being taken not to deposit polish material in the opening of the eggshell or needle. The needle is then inserted through the opening of the eggshell until the surface of the cork rests firmly on the eggshell. The cork is held in place on the egg by two narrow strips of Scotch tape until the nail polish has dried (Fig. 3).

11. The eggs are placed back in the incubator. The syringes are held by wooden clamp clothespins and the free end of the clothespins are placed in a test tube rack (Fig. 4).

12. Daily injections are given of various solvents for a period of seven days. On the eighth day the eggs are opened and examined.

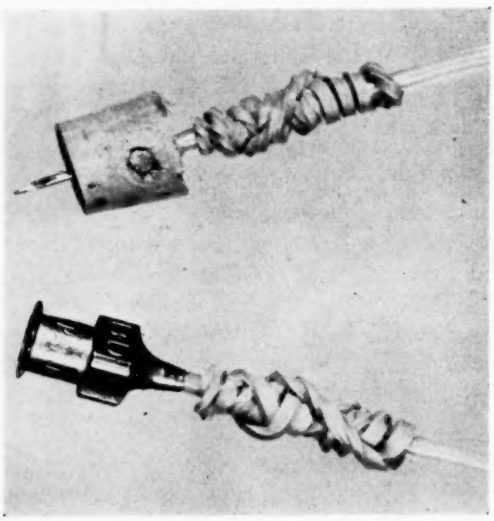

Fig. 1.—Close-up view of mounting of the two needles in the plastic tubing.

SOLVENTS TESTED

With the use of many chemical compounds several types of solvents are necessary if the proper solubility and concentration are to be obtained. With this in mind several commonly available solvents were evaluated in regard to their toxic effect on the developing embryo when injected in 0.05 ml. and 0.1 ml. amounts at daily intervals for a seven-day period. Table I summarizes the results of the solvents tested.

DISCUSSION

Although the method described above requires somewhat more time in the initial preparation of the experiment, this objection is offset by the ease in

which subsequent injections can be given and the minimal amount of time required. The eggs do not require manipulation with subsequent injections and apparently their development is not influenced by the presence of the needle in the chorioallantoic cavity. It has been found that, if the tip of the needle

Fig. 2.—Single assembly showing condom attached to tuberculin syringe allowing free motion of the barrel and not obscuring the calibration of the syringe.

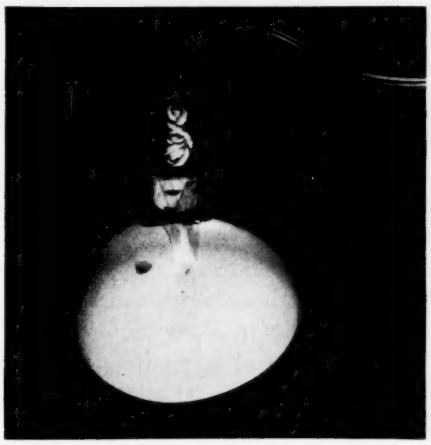

Fig. 3.—Close-up view of egg showing the attached cork and needle inserted into the chorioallantoic cavity. The Scotch tape is applied directly after the needle has been inserted and holds the cork and needle in place until the nail polish has hardened.

is extended 5 mm. beyond the lower surface of the cork, the material is deposited in the chorioallantoic cavity. There has been no instance in this series in which the needle has been occluded, although upon opening the eggs and examining the needle there is occasionally a whitish deposit around it. The cork is used to give support to the needle and to hold it firmly in place. There has been no instance in which the solvent has oozed out around the needle or cork. Once the nail polish has dried, the cork remains firmly attached to the eggshell, and the shell usually fractures elsewhere when an attempt is made to remove the cork from the egg. The 18-gauge plastic tubing is slightly larger than the shaft of a 20-gauge needle, and must be held to the shaft of the needle by a rubber band. The rubber band should be applied only tightly enough to accomplish this, because with subsequent autoclaving the plastic tubing tends to become soft and, if the rubber band has been applied too tightly, it tends to cut into the tubing and occasionally through it.

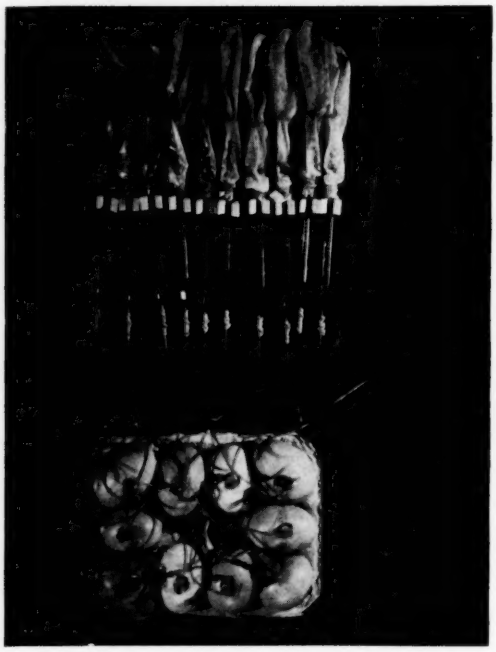

Fig. 4.—Complete assembly showing method of holding syringes and position of eggs in the incubator.

During autoclaving, care should be taken not to place any heavy object on the tubing or allow any sharp bend in the tubing, since during autoclaving the lumen is easily occluded. During autoclaving the tubing develops a milky appearance but this disappears within a few hours. After cooling the tubing is slightly more rigid. Autoclaving at 15 pounds pressure for twenty minutes minimizes the above noticed effects. The length of the tubing is optimal within certain limits depending upon whether the syringes are to be placed within the incubator or outside. In these experiments a length of two feet was used, the

TABLE I. SOLVENTS INJECTED DAILY INTO CHORIOALLANTOIC CAVITY OF TEN-DAY-OLD EMBRYONATED EGGS. INJECTIONS GIVEN DAILY FOR SEVEN-DAY PERIOD

SUBSTANCE	DAILY DOSE	NUMBER OF EGGS INJECTED	NUMBER OF EMBRYOS DEAD UPON OPENING	OBSERVATIONS
1. Distilled water	0.1 ml.	10	1	Normal embryos
2. Normal saline	0.1 ml.	10	0	Normal embryos
3. Olive oil	0.1 ml.	9	1	Oil droplets in the chorioallantoic cavity—embryos appeared normal
4. Sesame oil	0.05 ml.	9	0	Oil droplets in the chorioallantoic cavity—embryos appeared normal
5. Mineral oil	0.05 ml.	10	1	Few oil droplets in the chorioallantoic cavity—embryos appeared normal
Mineral oil	0.1 ml.	8	5	
6. Polyglycol E-200*	0.05 ml.	10	6	
Polyglycol E-200*	0.1 ml.	10	10	
7. Polyethylene glycol #400†	0.05 ml.	10	2	Embryos appeared normal—no trace of the polyethylene glycol found
8. Polyethylene glycol #600† (pH 4.62)	0.05 ml.	10	0	Embryos appeared normal—no trace of the polyethylene glycol found

*Polyglycol E-200, Texas Production, Texas Division, The Dow Chemical Company, Freeport, Texas.

†Carbide and Carbon Chemicals Division, Union Carbide and Carbon Corporation, New York, N. Y.

lumen of which required about 0.9 ml. to fill. This tubing can be reused after proper cleansing. It is apparently quite inactive chemically. The condom was used over the end of the syringe to prevent evaporation of the solvent in the syringe and bacterial contamination extending down along the barrel of the syringe. It may be used repeatedly.

Although contamination is a possibility due to the permanent insertion of the needle in the chorioallantoic cavity, this has not occurred in a single instance. It is apparent that the developing embryo can adjust to 0.1 ml. of an innocuous solvent given daily over a seven-day period without deleterious effects. The syringes may be placed inside or outside the incubator. However, the syringes should be placed at about the same level as the eggs. If this precaution is not observed there is an occasional incidence in which there is an excess of test substance deposited in the chorioallantoic cavity due to the siphoning effect of fluid in the tubing in those syringes with loose plungers.

Only a few solvents were evaluated for their toxic effects on the developing embryo. With the use of a one cubic-centimeter tuberculin syringe, daily injections into the chorioallantoic cavity of 0.05 ml. to 0.1 ml. are desirable since these quantities of fluid can be measured accurately. Of the solvents tested with 0.05 ml. amounts, only polyglycol E-200 was found to be highly toxic to the developing embryo. Mineral oil was found to be toxic in 0.1 ml. amounts but 0.05 ml. allotments were well tolerated. There are many other solvents which can most likely be injected into the chorioallantoic cavity without deleterious effects on the developing embryo. All of the solvents evaluated except

polyglycol E-200 had no visible effect on the developing embryo or its membranes. However, with olive oil, mineral oil, and sesame oil, small droplets of the injected material were seen in the chorioallantoic cavity upon opening the eggs. These droplets did not appear to provoke any reaction in the chorioallantoic membrane.

SUMMARY

1. A method is described for the multiple injection into the chorioallantoic cavity of embryonated eggs.

2. Several common solvents were evaluated for their effects on the developing embryo.

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SUDAN BLACK B IN MALE FERTILITY STUDIES

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THIS article presents a new approach to staining technique in fertility studies in man. It suggests the application of sudan black B, a lipophilic stain, as an adequate and, in many respects, preferable procedure in evaluating the morphologic and cytologic variations of spermia (spermatozoa).

Its application to fertility studies was conceived during investigations on the cytologic distribution of lipids within spermia and their antecedents.[1, 4] The microscopic observations that these lipids have differentially important distribution, and that the essential cytologic details for fertility evaluation are provided without the masking effect of massive staining of all seminal constituents usually encountered with other stains, led to its use, and to the conclusion that its further use is justified by merit. In evidence are the facts that the staining technique is rapid, involves only two simple steps in procedure, is economical, requires a minimum of equipment, and yet provides a clear differential picture (Plate 1).

HISTORY

Sudan black B, the most recent, and by some[1, 2, 3, 4, 7, 8, 10] considered to be the best of the lipophilic sudan stains available in cytochemical studies for the identifications of lipids, was first used by Lison (1936)[8] as a histologic stain. Leach (1938)[7] introduced diacetin (diluted with distilled water to a doubled volume) as a solvent substitute for the 70 per cent alcohol. The diacetin sudan black B stained his preparations more slowly, evaporated less during observations, and did not bring the lipids into solution.[7] Ethylene glycol also has been used advantageously by Hartman.[2] They studied wet preparations. Burton and associates[3] used heat-fixed dry films with counterstain for their studies on lipids in spore-forming bacilli.

RESULTS

The use of sudan black B as a differential stain has no recorded precedent. It nevertheless does serve well in the morphologic and cytologic evaluation of spermia in fertility studies. It stains only the lipids a blue-black color. Consequently, the semen, with its constituent proteins, salts, sugars, and water, remains unstained except for the lecithin and cholesterol bodies and other staining units derived from the cytoplast remnants, e.g., mitochondria, Golgi remnant, microsomes, and cell wall parts, which were discarded into the seminiferous tubules during the metamorphosis of the spermatid to the spermium.[1, 4] The matrix (seminal constituents) which surrounds the spermia, therefore, does not stain, nor does it in any way obscure the microscopic picture of the individual spermium and its parts. They, in contrast to the matrix, and, due to their lipid content, stain in every detail essential to their morphologic evalua-

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tion (Figs. 1 to 12). The stained cell wall surrounding the *head* delineates its size, shape, position and structural elements. The nucleus stains faintly but differentially. The *body* (middle piece) contour is much in evidence due to the lipid content of the wall and the organelles found therein, e.g., the anterior and posterior end knobs, the so-called spiral filament, and the end ring, all of which stain darkly. Any cytoplasmic residual adhering to the middle piece is outlined without obscuring details of underlying structures. Similarly, in the *tail* the axial fiber of the flagellum stains well throughout its length, making duplication or variants readily recognizable.

TECHNIQUE OF PREPARING SLIDE

The slide is prepared by making a *film* of acceptable ejaculate,[5] *fixing* in Schaudinn's fixing fluid (cold-saturated aqueous $HgCl_2$, 66 ml.; 95 per cent alcohol, 33 ml.; glacial acetic acid, 1 ml. mixed with above alcoholic mercuric chloride just before using) and *staining* in sudan black B alcoholic solution. The stain is prepared by adding to 70 per cent alcohol enough sudan black B powder to saturate it (0.3 Gm. stain to 100 ml. alcohol). More concentrated alcohol may be used advantageously to dissolve the powder, but the concentration must be reduced to 70 per cent before applying to cells. Concentrations above 70 per cent will remove the lipid cell constituents. Ethylene glycol, a dihydroxy alcohol, may be substituted for ethyl alcohol. The stock stain, if 70 per cent, is allowed to stand for at least twenty-four hours before using, and is applied to the cells at least one-half hour (warmed) to twenty-four hours before they are conclusively studied.

The stain will form a fine precipitate after standing for several days. For economy and for best staining, slides are, therefore, immersed with the cell surface down and with one end of the slide elevated in the vessel containing the stain; the precipitate in response to gravity will thus be much less likely to accumulate upon the cells. If unprecipitated or filtered staining solution is used, the cells may be flooded with stain, covered with a slip, and immediately placed under observation until desired staining depth is obtained. Both procedures are equally good provided the solvent is saturated with the sudan powder, and cell surfaces are covered by the solution at all times. This last provision requires vigilance when using ethyl alcohol wet-mounts, as there is rapid evaporation of its thin film during microscopic observation. Wet mounts do expedite examination as they can be observed with oil immersion objective early by covering the stain-flooded cell area with a cover slip. Further, conclusive evaluation of the cells present may be done without mounting. If a more thorough microscopic study is planned, or a more convenient time desired, clear Karo serves most satisfactorily as mounting media. The slip edges may be sealed for semipermanency. Heat-fixed, dry slides are less satisfactory for spermia than for bacteria[2] or pus.[9]

DISCUSSION

The staining response of sudan black B is due to its lipophilic qualities and not to any ionic combination, e.g., esterification or other chemical union. The stain enters the cell and remains in solution differentially through its greater solubility in the lipid-containing cell structures. Destaining is accomplished through application of its solvents.

Procedures entailed in the usual dehydrated and cleared slide cannot be applied to spermia nor to their tissue progenitors as their intracellular lipids, which take the stain, are dissolved out by these fat solvents. The sudan black B stain is removed during the dehydrating and clearing processes wherein the more concentrated alcohols and xylol are used. Mounting fluids containing

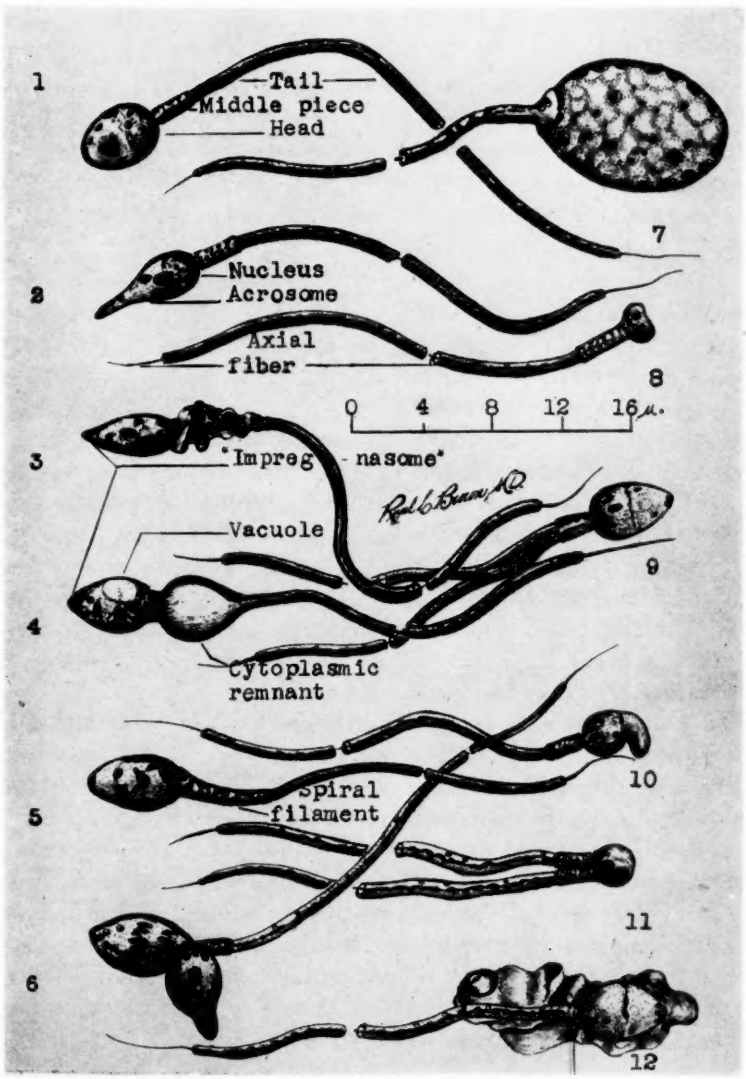

Plate 1.—Normal and abnormal spermia stained with sudan black B.

Normal: Fig. 1, Frontal view. Fig. 2, Lateral view.

Abnormal (may or may not accomplish impregnation): Fig. 3, Incompletely shed cytoplasmic remnant. The remnant appendage appears collapsed with no evident endoplasmic contents. Fig. 4, Appendage from cytoplasmic remnant intact and attached to middle piece. Head contains large acentric vacuole primarily in nuclear area. Fig. 5, Elongate middle piece; spiral filament in evidence. Fig. 6, Dicephalic form. Fig. 7, Megalocephalic form. Fig. 8, Microcephalic form. Fig. 9, Dicaudid form with single broad middle piece. Fig. 10, Distorted (flexed) acrosome. Fig. 11, Microcephalic form with duplication of middle piece and tail. Fig. 12, Normal spermium with fragmented cytoplasmic remnant apparently composed only of incompletely shed cell wall.

All figures drawn (camera leucida) from sudan black B stained spermia present in semen from fertile man.

All forms contain numerous lipid or lipid-containing bodies which stain with lipophilic sudan black B. The apex of the acrosome contains the so-called impregnosome.[1, 3]

these or other fat solvents produce similar effects. Thus the conventionally cleared and mounted slides, following sudan black B staining, cannot be prepared.

Destaining for further study in which other stains and procedures are desired is accomplished very easily by exposing the sudan black B stained cells to alcohols above 70 per cent concentration, either as a destaining or a dehydrating process. A smear or cell preparation can thus be treated as specified for any other staining procedure or other technique in which the lipid elements are of no immediate concern.

The staining processes of sudan black B can be expedited, if desired. Warming the sudan staining solution during staining will accomplish this. Accelerated evaporation of the wet mount while under light sufficient for microscopic observation may obviate the ultimate facility in such technique. For semipermanent mounts, warm stain is superior. The fixing and staining procedures also can be combined by saturating the Schaudinn's fluid with the sudan black B powder twenty-four or more hours before using. The heating of the Schaudinn's fluid-dye will further expedite the staining procedures. Furthermore, sudan black B powder or solution can be mixed into other alcoholic (70 per cent) stains, e.g., iodine, Giemsa,[9] etc., for counter or special staining effects thereby eliminating the time essential to a second procedure. If desired, the slide stained with counterstains may then be mounted permanently. In this event the lipids and sudan staining structures will be lost to the dehydrating alcohols and clearing agents.

Sudan black B is easy to work with. Slides can be retained in the 70 per cent alcoholic or ethylene glycolic sudan black B indefinitely with improved staining, provided the stain remains effective and the lipids remain in situ. Stain and precipitate can be removed from the cells fairly well by returning the slide to 70 per cent ethyl alcohol for twenty-four hours or more. Errors and mishaps are corrected with any of its solvents, preferably 70 to 100 per cent alcohol or xylol. Furthermore, it may be used as a primary stain, as enough of the characteristic cytologic structures of most cells are stained by sudan black B to make them identifiable without counter stain. Consequently, it alone may be used advantagenously as a rapid, differential stain in the morphologic evaluation of spermia in fertility studies.

SUMMARY

1. Sudan black B is frequently a preferred stain in fertility studies.

2. It colors blue-black the lipid constituents of the spermia.

3. It gives the cytologic details desired without obscuring any of the cells or their structures.

4. The invariable distribution of lipids within evaluatively important cell structures and its comparative absence in semen makes possible a marked differential picture.

5. It is rapid, economical, requires a minimum of apparatus, chemicals, and procedures, and yet gives evaluatively adequate results.

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A DIRECT READING DIFFERENTIAL PRESSURE FLOW METER, COMPOSED LARGELY OF COMMERCIALLY AVAILABLE COMPONENTS, AND HAVING A LINEAR CALIBRATION

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A LARGE number of flow meters which employ the principle of recording the drop in pressure along a flowing stream have been described in the literature dealing with hydraulics, physics, and physiology.[1] In most cases the system for recording the drop in pressure has been relatively insensitive, so that some structure had to be inserted in the path of the flowing stream in order to increase the pressure drop. Devices which have been used for this purpose include the following: (a) A constriction is built in the flow tube, which is designed to increase the frictional resistance to flow. (b) A Venturi tube is utilized in which the flow tube somewhat abruptly narrows, and then enlarges gradually to the original diameter. Since the velocity is greater in the smaller segment, the lateral pressure is lower here than in the larger upstream segment, and this difference increases with increased flow. (c) An orifice meter is used which employs a disc with a central opening smaller than the tube diameter inserted in the path of the flowing stream. The resultant increase of velocity lowers the lateral pressure downstream in relation to the lateral pressure upstream from the orifice. (d) A Pietot tube is inserted into the flowing stream. One arm is directed upstream and the other arm is directed downstream or at a right angle to the direction of flow. The pressure is greater in the upstream tube.

These devices have the common feature that they diminish at some point the diameter of the flow tube. It results, therefore, that two independent variables, frictional resistance and velocity, contribute to the differential pressure which is created. As a consequence, the differential pressure is a nonlinear function of flow. In most cases, the differential pressure is related to the flow as indicated in the equation:

$$P = K_1 \cdot F + K_2 \cdot F^2$$

Where P represents the differential pressure, K_1 and K_2 are constants related to the meters used, and F represents flow. K_1F is the contribution of frictional resistance, and K_2F^2 is the contribution of velocity in the equation.

Prevalent devices used for recording the magnitude of differential pressure include: (a) water or other fluid manometers, (b) bellows systems for operating

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writing pens, and (c) optically recording differential pressure manometers. The latter have the proper combination of high natural frequency and sensitivity to record the transients in physiologic pulsating streams, but incur difficulty in critical damping, are difficult to construct, and tend to deteriorate rapidly. While the fidelity of such optical meters is adequate if properly adjusted, these meters always are nonlinear and require redrawing the flow curves with a linear ordinate scale before integration is performed. At best

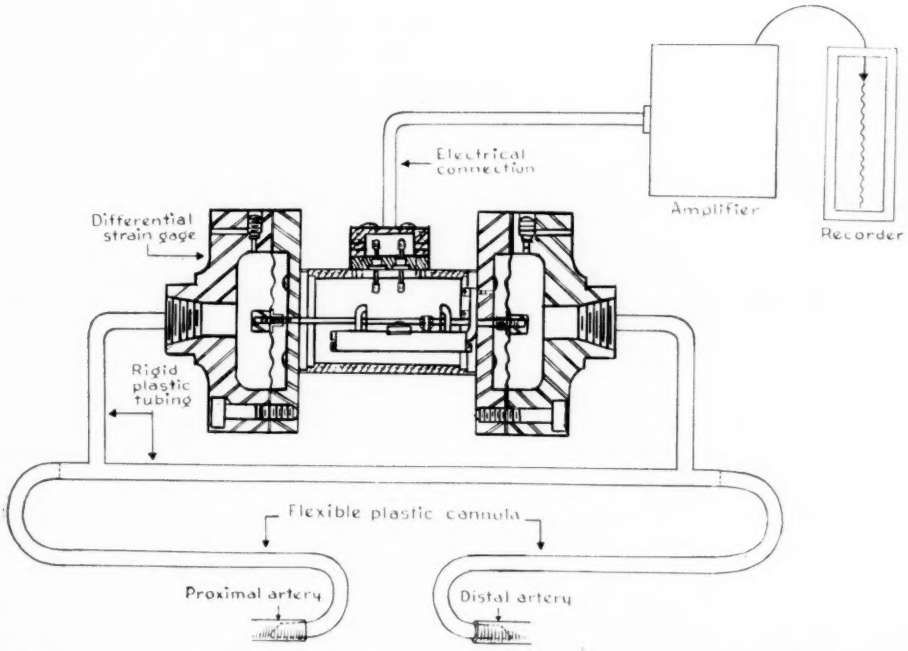

Fig. 1.—Schematic diagram of differential pressure friction flow meter showing the differential strain gauge and the method of cannulation.

these meters require 5 to 10 mm. Hg differential pressure for full scale deflection. This pressure loss plus that in the associated connecting tubes results in a considerable drop in the perfusion pressure in the artery where flow is being measured. Furthermore, the meters are critically sensitive to changes in diameter, and any deposits of platelets or fibrin cause a considerable shift in calibration.

The present paper describes an electrically operated direct writing system of simple construction with no constriction in the tube for pressure drop and which requires less than 3 mm. Hg pressure loss in the blood stream at 60 ml. per minute. It has a sufficiently high natural frequency to follow with fidelity the transients of physiologic pulsating streams, and is conveniently adjusted to a wide range of sensitivities. With no constriction in its lumen, the differential pressure created is relatively a linear function of flow, since it employs the single independent variable, frictional resistance as its principle.

DESCRIPTION OF EQUIPMENT

The apparatus consists of a flowmeter tube (Fig. 1), a Statham differential strain gauge,* a Brush strain analyzer, and a Brush magnetic recorder.†

The flowmeter differential pressure pickup is constructed from a rigid polystyrene tube 30 cm. long with a uniform 3 mm. bore. The pressure drop due to friction between the two lateral piezometer connections, 26 cm. apart, is transmitted through two arms of the same material to opposite sides of the differential strain gauge. The pressure loss in the flow stream of this tube under physiologic conditions is less than 3 mm. Hg at 60 ml. per minute blood flow, and is 50 per cent of this value at 30 ml. per minute blood flow. Measurements show the pressure loss with saline as a fluid to be approximately one-fifth of these values.

The strain gauge is a Statham model P79-0.5D-840 which is not sensitive to pressures applied equally to both sides and, therefore, is not affected by arterial pressure. However, it is highly sensitive to differential pressures created by flow through the flowmeter tube. This gauge with the inherent fluid system described is not critically damped, but critical damping may be achieved readily while recording by minor modifications of the strain analyzer as described below.

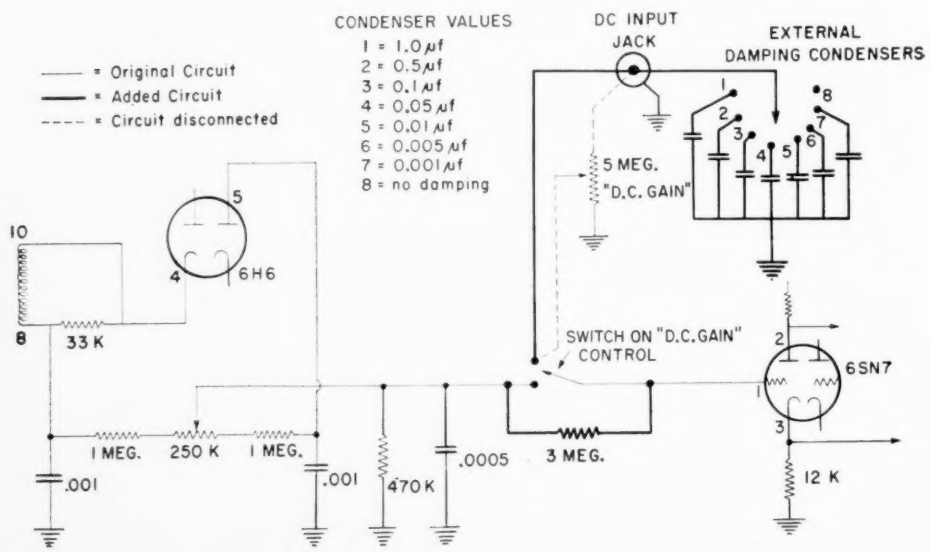

Fig. 2.—Diagram demonstrating a suggested modification of the analyzer circuit designed to allow critical damping or overdamping of transients.

The Brush strain analyzer supplies a 2,000 cycle carrier wave voltage to the strain gauge, where it is imposed upon a bridge circuit. The analyzer amplifies the forcing potentials generated as pressure on the gauge unbalances the bridge, and the voltages are amplified, rectified, and transmitted to the magnetic pen recorder. Because of a discriminator built into the analyzer, forward and backward flows are differentiated and faithfully recorded. By the insertion of a three megohm resistor in series with the circuit just preceding the D.C. amplifiers, and by the use of selectable condensers which may be plugged into the "D.C. input" jack (Fig. 2), critical damping may be obtained. One also may overdamp the recording with a larger condenser to integrate the transients and obtain a record of mean flow. If fidelity of pulse contour is not a requirement, a slower-responding recorder such

*Statham Laboratories, Inc., 9328 Santa Monica Blvd., Beverly Hills, Calif.
†The Brush Development Co., 34 Perkins Avenue, Cleveland 14, Ohio.

as an Esterline-Angus 5 ma. 70 ohm type* may be used without critical damping. This type of recorder is quite adequate for mean flow measurements where the slow pen speed supplies damping of the pulse contour. Such a recorder possesses adequate sensitivity, but a matching resistor of approximately 1,500 ohms should be inserted in series with the movement to protect the instrument. A Sanborn† or other D.C. recorder also may be used with the strain analyzer, if more convenient.

RESULTS

Characteristics.—Fig. 3 shows a typical calibration curve of the instrument using a Brush recorder with the analyzer gain setting at attenuation 100. Step-

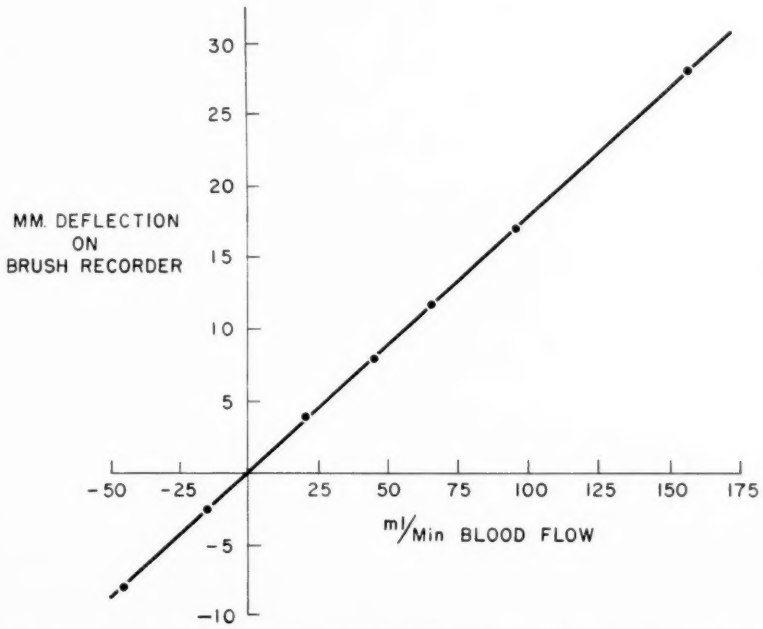

Fig. 3.—Typical calibration curve of the differential pressure flow meter system, with the analyzer gain setting on attenuation 100 as reproduced on a Brush magnetic recorder.

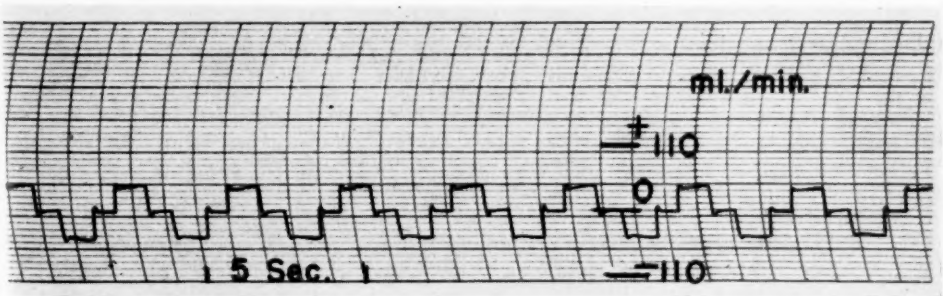

Fig. 4.—Reproduced contours of flow created by a hydraulic square wave generator, with the analyzer slightly underdamped, demonstrating the fidelity of the system in recording forward and backflows of similar magnitude.

*Address: P. O. Box 596, Indianapolis, Ind.
†Address: 39 Osborn St., Cambridge 39, Mass.

wise gains up to five times this magnitude (attenuation 20), and lower gains down to 5 per cent of this magnitude (attenuation 2,000), are possible with stability. The calibration curve is always linear within less than ± 10 per cent error up to full scale with both forward and backward flows, so that flow in either direction may be measured adequately in a pulsatile stream. It may be seen that 175 ml. of blood per minute gives full-scale forward deflection at this setting. At this flow the pressure drop is 7 mm. Hg.

Reproduced contours of flow induced by a hydraulic diphasic square wave generator are represented in Fig. 4. This square wave generator consists of a 10 c.c. syringe connected directly to the flowmeter tube. The plunger of the

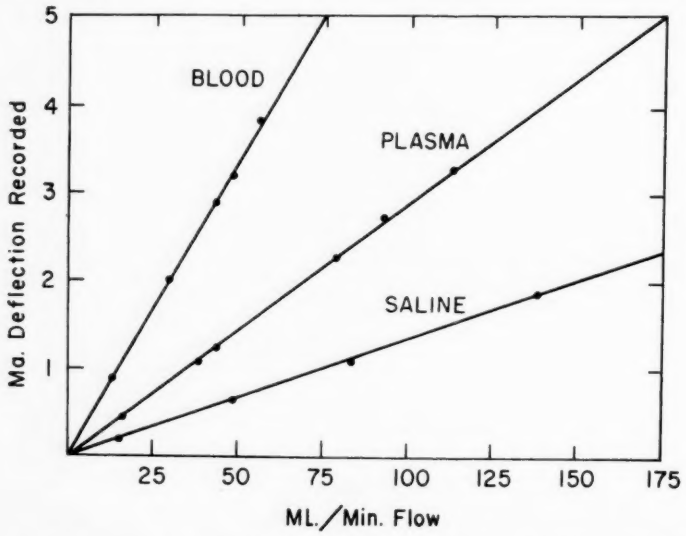

Fig. 5.—Milliamperes deflection on an Esterline-Angus 5-milliampere 70-ohm recorder as a function of blood, plasma, and saline flow, demonstrating the effect of physiologic systems of different viscosities.

syringe is attached to a dual set of racks between which is a cog with three-fourths of its teeth removed. When the cog is rotated the teeth engage one of the racks during one-fourth of the rotation, are free during the second quarter, engage the second rack during the third quarter, and are free again during the fourth quarter of rotation. This results in an abrupt constant flow in each direction alternately, with an intermission of no flow. It may be observed that both forward and backward flows of equal magnitude are recorded with close fidelity. The instrument in this case is near-critically damped for optimal response.

Routine tests of pressures exerted on the two sides of the gauge simultaneously, whether momentary or prolonged, cause a pen deflection of less than 1 per cent at 150 mm. Hg pressure, which may be interpreted as less than 1.5 ml. per minute, flow error at attenuation 100, under physiologic conditions. The natural frequency of the system when filled with physiologic saline is approximately 20 cycles per second.

Since this type system is sensitive inherently to viscosity, studies were made of the effect of filtered whole blood, of plasma, and of 0.9 per cent saline upon the calibration of the instrument. The deflections due to these fluids are shown in Fig. 5. It may be seen that the apparent viscosity of blood is approximately

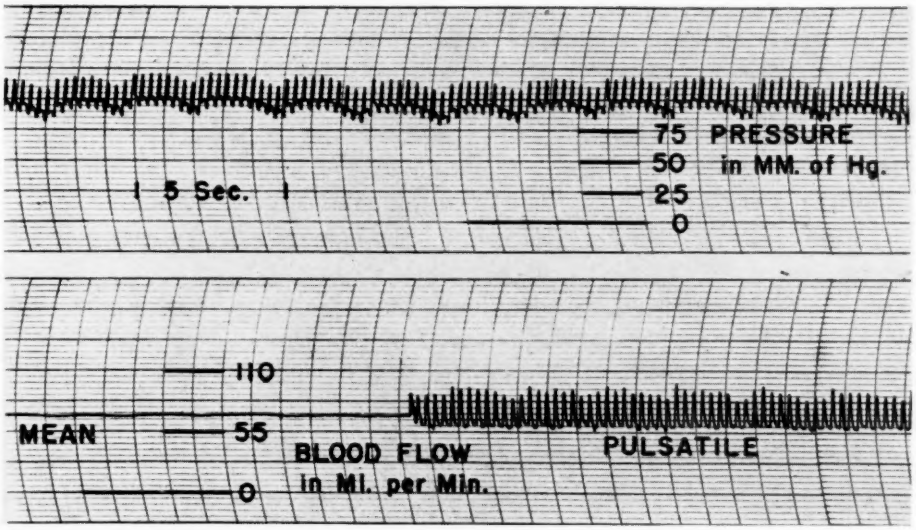

Fig. 6.—Pressure and blood flow records taken from the femoral artery of the dog. The flow record is heavily damped in the first half of the record and moderately damped in the second half to show mean flow electronically integrated by capacitance (Fig. 2).

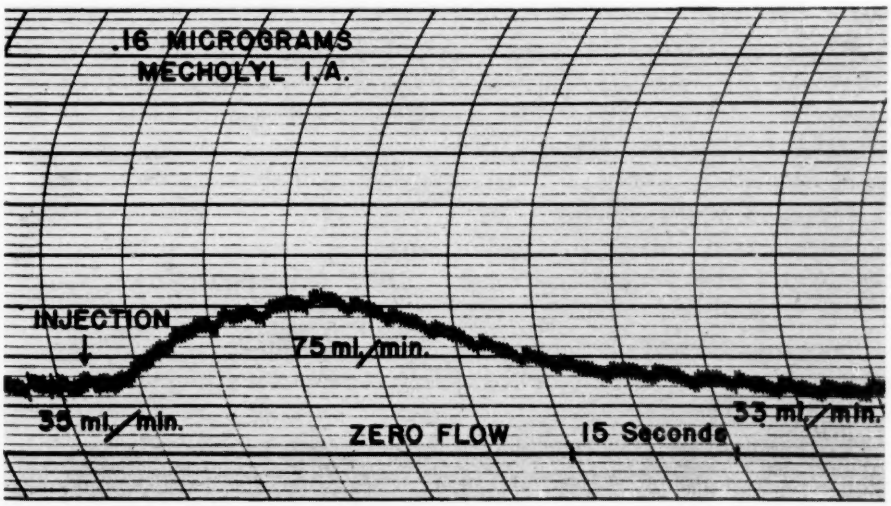

Fig. 7.—A sample record of blood flow changes in the femoral artery of a dog following an intra-arterial injection of Mecholyl (methacholine), as reproduced on a 5-milliampere recorder.

five times that of saline, whereas the apparent viscosity of plasma is 42 per cent that of blood. These data demonstrate that a change in hematocrit will change the calibration to a predictable value.

Calibrations were taken approximately hourly over a four-hour period while measuring flow in the femoral artery of the dog, and calibrations were taken from various animals daily. The calibrations varied less than 10 per cent, which is within the range of error of the instrument. However, due to possible hematocrit changes of greater magnitude, frequent calibration checks may be recommended to exclude errors due to viscosity changes.

Fig. 6 shows records of blood flow in the femoral artery of a dog, demonstrating pulsatile flow registration, along with the same flow integrated by the damping of a 1.0 Mfd. condenser (Fig. 2) to register mean flow in another segment of time. The record also includes the arterial blood pressure, which may be utilized along with the flow data to compute the peripheral resistance.

Fig. 7 demonstrates the effect in the femoral artery of the injection of 0.16 micrograms of methacholine upon blood flow as measured with this flow meter with an Esterline-Angus recorder. The methacholine was injected intra-arterially at the point with time delineated on the record. Both the height of response and the duration of response may be evaluated quantitatively in detail.

DISCUSSION

Care should be exercised in balancing the bridge of the instrument, especially the capacitance control of phase, in order to avoid distortion of the calibration curve or a shift of the apparent zero. When this precaution has not been observed carefully, calibration shifts up to 10 per cent have been found. With care, zero shifts may be kept below 3 per cent over a four-hour period, with no observable change over shorter periods.

There are certain advantages in the use of polystyrene tubing in the flowmeter tube. It presents a relatively nonwettable surface to be in contact with the flowing blood, thereby reducing the anticoagulant required to prevent fibrin formation. Its rigidity prevents damping of the pulse contour, and assures the maintenance of a constant distance between the piezometer connections during operation, which is necessary for a constancy of calibration. This plastic is managed easily in the construction procedure, making a permanent bond when coated with toluene as a solvent. Other nonwettable plastic materials may be found equally suitable.

The adaptation of an integrating circuit to damp electrical voltage transients makes it possible to record pulsatile and mean flows alternately or simultaneously by the use of two analyzers, thereby allowing a true average flow to be available to scrutiny while observing the pulse contours in detail.

While cannulation of the vessel being studied, and the use of anticoagulant, such as heparin, is necessary with the the use of this blood flow meter, it is believed by this group that the instrument as described possesses a maximum of desirable features and is a fundamental improvement theoretically and practically over previous types of differential pressure blood flow meters. It has the further advantage of being constructed entirely of commercially available components with the exception of the flow meter tube.

SUMMARY

A new type of instrument has been developed for the measurement of pulsatile and mean blood flow, which uses the principle of differential pressure created by the friction of a flowing stream. It does not use a decreased lumen-diameter, and therefore has a linear calibration within 10 per cent error to full scale and a pressure loss of less than 3 mm. Hg at 60 ml. per minute. It is simple to construct and readily adaptable to use in the experimental laboratory.

Paul E. Moore, Gwen Roberts and Nancy C. Kester assisted in the development and improvement of this instrument.

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ENZYME ASSAY WITH A COAXIAL VISCOSIMETER

I. Description of the Instrument

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CLINICAL study of pancreatic physiology and diagnosis of pancreatic disease have suffered from lack of rapid and accurate methods for estimating the activity of the external secretions of the pancreas.[1] Most of the chemical methods for determining amyloclastic and tryptic activity are tedious and subject to rather wide variations. In 1923, Northrup and Hussey[2] reported that tryptic activity could be quantitatively measured with Ostwald viscosimeters and, in 1925, Davison[3] reported assaying the amyloclastic activity of duodenal contents in the same way. Viscosimetric assay of the amylase activity of blood serum soon followed, and it was shown that the reaction between enzyme and substrate follows Arrhenius' "Q × T" rule when the time to produce a given decrease in viscosity is observed.[4-7] The principal objection to use of an Ostwald viscosimeter is the serious effect slight temperature variations have upon the results. This, and other technical difficulties, led even proponents of this viscosimetric method to discourage its use for routine, clinical assay of amyloclastic activity.[7] However, when conditions were rigidly controlled, the method was accurate to within 10 per cent in tests on duodenal contents[6] and blood serum,[8] and had a reliability similar to that of saccharogenic tests on blood serum.[9] Viscosimetric assay of trypsin was reported to be less accurate,[6] but part of the variability was believed due to the instability of this enzyme.

In this laboratory, work with a Lecomte du Noüy microviscosimeter indicated that a similar, but less delicate, instrument might serve to record the viscosity changes of a substrate during enzymatic action. Although coaxial viscosimeters have been used since 1847 and their theoretical advantages over capillary viscosimeters described,[10, 11] they apparently have not previously been used to measure the speed of enzymatic reactions. Therefore, an experimental instrument was constructed, and its applicability to assays of amyloclastic and tryptic activity investigated with the view of developing simple, rapid, and accurate methods suitable for clinical and commercial laboratory use.

The great need for such methods has prompted this preliminary report in the hope that it will stimulate further development of methods.

INSTRUMENT

The basic principle of the coaxial viscosimeter is that a revolving fluid mass transmits force, as torque, to a solid object or pendulum immersed within it. If the fluid mass is revolved at a constant speed, and its temperature is constant, the amount of force trans-

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mitted to the pendulum will vary directly with the viscosity of the fluid, provided the speed is not too great. By measuring the force exerted on the pendulum, or the counterforce necessary to prevent its rotary movement, a measure of relative viscosity is readily obtained. If the counterforce necessary to "neutralize" rotation of the pendulum can be read continuously, changes in the viscosity of the rotating fluid can be determined instantly. The sensitivity of a coaxial viscosimeter depends upon several interrelated factors. In the present instrument, they are adjusted as follows: (1) rate of rotation—300 r.p.m.; (2) clearance between cup and pendulum—$\frac{1}{16}$ to $\frac{1}{8}$ inch; (3) pendulum size and buoyancy—solid aluminum body, $\frac{5}{8}$ inch by $\frac{1}{4}$ inch cone plus $\frac{5}{8}$ inch by $\frac{3}{4}$ inch cylinder, with a steel shaft, $\frac{3}{32}$ inch by $1\frac{3}{8}$ inches; and (4) method of supplying the counterforce—tachometer spring of fixed resistance. The pendulum dimensions are perhaps the most easily varied. The other dimensions or the arrangement of the instrument are not critical as long as perfect vertical alignment of the shafts of the recording device, pendulum, and motor is obtained.

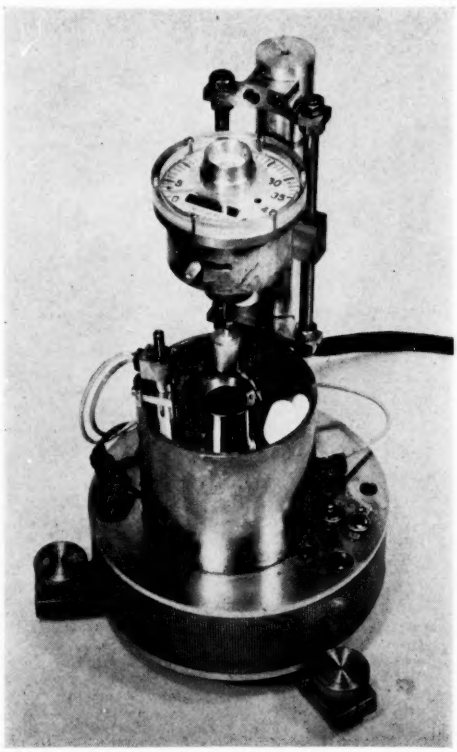

Fig. 1.—View of the viscosimeter with pendulum in raised position and cup on turntable.

The details of the instrument are shown in Figs. 1, 2*A*, and 2*B*. It is simply designed, combining machined parts with stock electrical materials and hardware. The removable turntable locks over a peg on the shaft of the synchronous motor (Merkle-Korff Gear Co., Chicago, Model SG10, clockwise drive). A skirt on the turntable rides in a well of mercury. This provides a relatively effective and frictionless seal between the surrounding oil bath and the motor. The cup, with an eccentric peg in its base, fits on the turntable, and is easily removed for cleaning. It has an operating capacity of 8.0 to 8.5 milliliters. Intermittent heat is supplied to the oil bath by two 7-watt light bulbs connected to a thermo-

regulator (Fenwal, Inc., Cat. No. 17200). An auxillary heater (heating unit from an old soldering iron) provides rapid heating when needed. The wiring within the oil bath is bare copper wire strung with glass beads. Short circuits will not occur if the mineral oil is changed when noticeable amounts of water or dirt accumulate in the bottom of the bath. Both heating circuits (inset, Fig. 2, *B*) contain pilot lights. Preferably, the power supply for the motor is monitored by a constant voltage transformer (minimum capacity, 0.5 ampere).

The recording device, from which the pendulum is suspended, travels on vertical rails and may be locked in the upper position when the cup and pendulum are to be cleaned. The jewel-mounted staff and spring assembly, dial and pointer from a tachometer (Stewart-Warner, No. 91150) are centered directly over the motor shaft. A yoke, with a slot and set screw for rigid attachment of the pendulum, is connected to the staff just above the lower bearing. A leveling bubble is mounted in the center of the lucite face of the recording device, and there are leveling screws in the legs of the instrument.

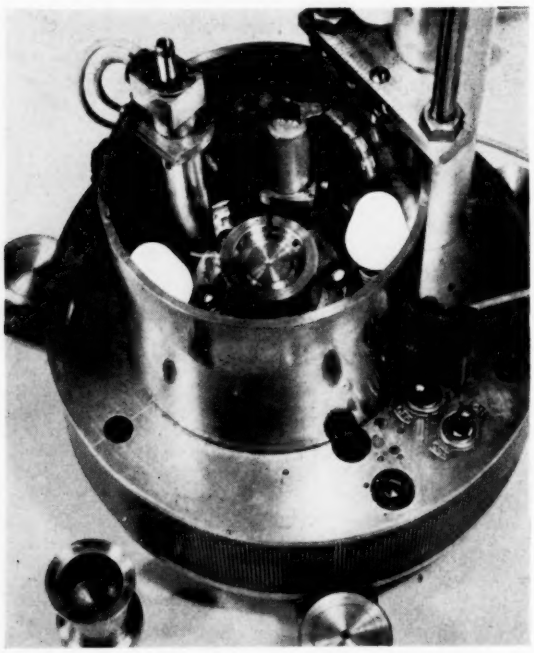

Fig. 2*A*.—Close-up view of the oil bath showing turntable (center), thermostat (upper left), which is connected to 7-watt light bulbs (lower right and left), and the auxilliary heating unit (upper right).

In a research instrument, an electrical recording system would be advantageous. Either replacing the spring with an armature operating in a magnetic field[11] or using a fixed pendulum and measuring the power required to maintain a constant motor speed[12] would provide such a recording system. Either system would adapt the apparatus to a wider range of substrate viscosities.

To determine the enzymatic activity of an unknown substance, sufficient substrate to cover the pendulum is placed in the cup and the motor started. After a 2- to 3-minute interval for temperature equilibration, the deflection of the pointer is recorded as the initial viscosity of the substrate. With the motor still running, the unknown substance is added to the substrate and, as digestion progresses, the decrease in viscosity is indicated by the gradual return of the pointer toward zero.

RESULTS

In preliminary experiments, 3 per cent rice starch paste and 15 per cent gelatin solutions were found satisfactory as substrates for the determination of amyloclastic and tryptic activity, respectively. Since relatively satisfactory chemical methods for lipase determinations are already in use, no especial effort has been made as yet to develop a suitable substrate for this enzyme.

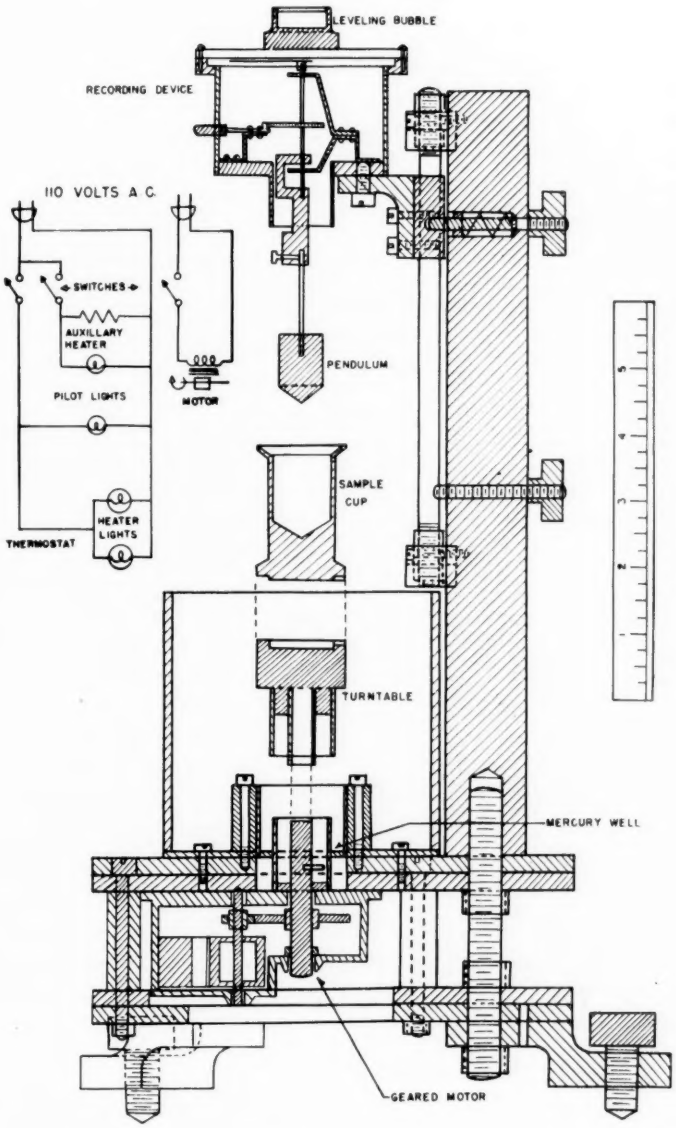

Fig. 2*B*.—Longitudinal right section of the viscosimeter and wiring diagram (inset) for the motor and heating circuits.

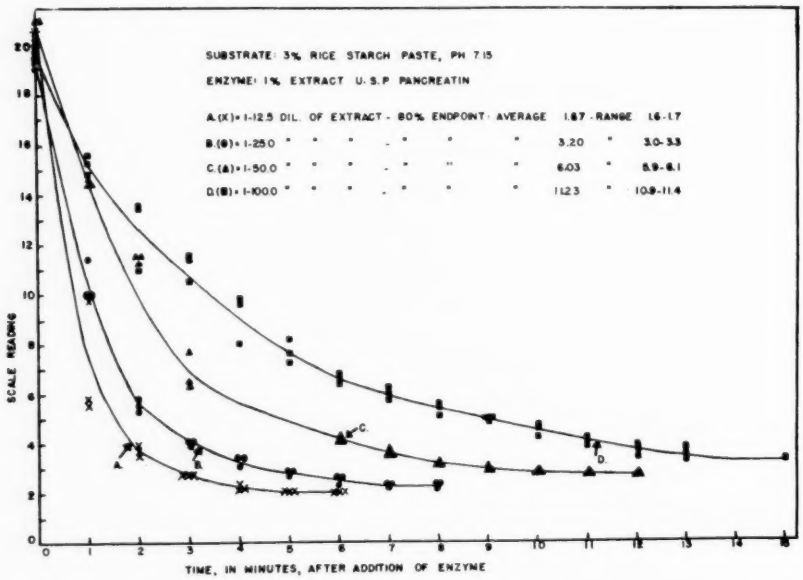
SUBSTRATE: 3% RICE STARCH PASTE, PH 7.15
ENZYME: 1% EXTRACT U.S.P PANCREATIN
A.(X)= 1-12.5 DIL. OF EXTRACT - 80% ENDPOINT: AVERAGE 1.87 - RANGE 1.6-1.7
B.(0)= 1-25.0 " " " " " " 3.20 " 3.0-3.3
C.(Δ)= 1-50.0 " " " " " " 6.03 " 5.9-6.1
D.(⊞)= 1-100.0 " " " " " " 11.23 " 10.9-11.4
SCALE READING
TIME, IN MINUTES, AFTER ADDITION OF ENZYME

Fig. 3.—Course of the reaction between starch substrate and various amounts of amylase. Each line represents the average of three determinations.

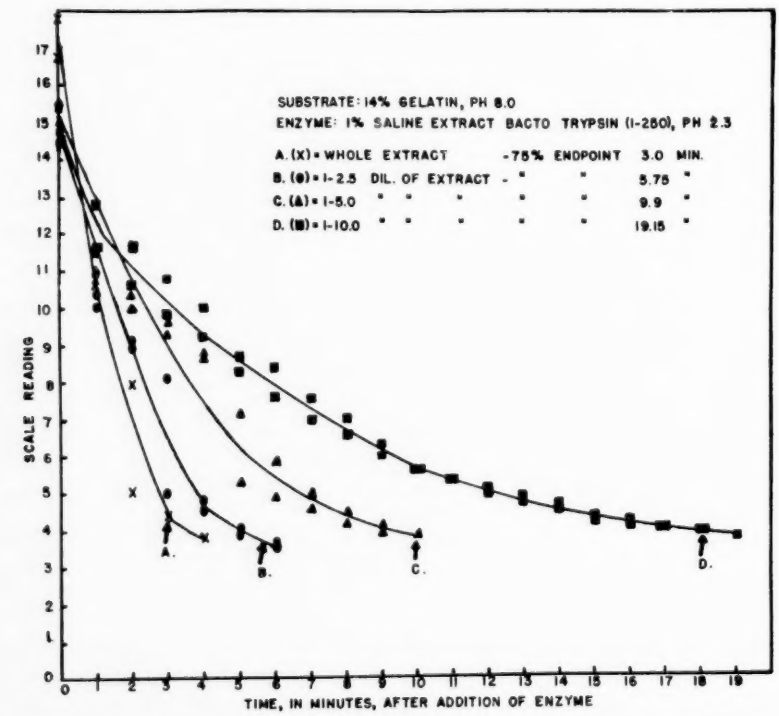
SUBSTRATE: 14% GELATIN, PH 8.0
ENZYME: 1% SALINE EXTRACT BACTO TRYPSIN (1-250), PH 2.3
A. (X) = WHOLE EXTRACT -75% ENDPOINT 3.0 MIN.
B. (0) = 1-2.5 DIL. OF EXTRACT - " " 5.75 "
C. (Δ) = 1-5.0 " " " " " 9.9 "
D. (⊞) = 1-10.0 " " " " " 19.15 "
SCALE READING
TIME, IN MINUTES, AFTER ADDITION OF ENZYME

Fig. 4.—Course of the reaction between gelatin substrate and varying amounts of trypsin. Each line represents the average of two determinations.

When amounts of amylase, which produced about 7,500 mg. glucose per 100 ml. in a saccharogenic test for amylase activity, were tested, the curve obtained by plotting viscosity against time (Fig. 3, line *A*) showed a rapid drop in the viscosity of the substrate during the first two minutes after the amylase was added. The reaction approached equilibrium in five to six minutes. With smaller amounts of amylase, the rate of viscosity change was decreased accordingly (Fig. 3, lines *B*, *C*, *D*). Similar curves were obtained when the reaction of trypsin with gelatin was plotted (Fig. 4).

Curves of the types shown in Figs. 3 and 4 were analyzed for points which would give reliable readings in the shortest possible time. In the present study, relatively large quantities of enzyme were employed. An 80 per cent decrease in viscosity was adopted as the end point for amylase, and 75 per cent was adopted as the end point for trypsin determinations. End points were obtained in three to fifteen minutes with 0.5 ml. specimens of enzyme which produced 700 to 3,600 mg. glucose per 100 ml. in saccharogenic tests for amylase activity. Specimens containing 100 to 500 μg. of crystalline trypsin yielded end points within the same time range. More active specimens were diluted until end points in this time range were secured. In viscosimetric testing, a lesser degree of digestion is advantageous when an unduly long time is required to reach the usual end point.[3] Tentatively, a 40 per cent end point has been adopted for studies on the amyloclastic activity of weakly active substances such as normal human blood serum.

DISCUSSION

When enzymatic reactions are studied with Ostwald viscosimeters, one or two minutes are required for each reading and for the return of the sample to the upper chamber, and the reaction is progressing as the reading is made. Thompson and associates,[13] who developed very precise methods for assaying amyloclastic activity with these instruments, used end points of not less than thirty minutes. In addition to its ruggedness, the outstanding advantage of the coaxial viscosimeter is that continuous, instantaneous readings are possible. This permits more accurate observation of the exact time at which the end point is reached, and substantially reduces the minimum end point time which gives reproducible results. Too, it allows closer study of the course of enzymatic reactions.

The data obtained with the coaxial viscosimeter on the course of the viscosity changes in a substrate during amyloclastic and tryptic reactions agree with that reported by others using Ostwald viscosimeters.[2, 4, 6, 9] Considerably greater concentrations of substrate and enzyme were used with our instrument, and this appeared to lessen the variability in duplicate tests caused by minor variations in temperature. For determinations of amyloclastic activity, control of the temperature to ± 0.2 to $\pm 0.3^\circ$ C., rather than to the $\pm 0.01^\circ$ C. recommended for the pipette technique,[5] was sufficient.

Methods for assay of the amyloclastic activity of some substances will be reported separately. Those for assay of tryptic activity have not yet been completely developed.

SUMMARY

A coaxial viscosimeter, designed to meet the needs of clinical and commercial laboratories for simple, rapid, and accurate methods of enzyme assay, is described.

Data on amyloclastic and tryptic reactions comparable to that reported for Ostwald viscosimeters were obtained under conditions readily attainable in small laboratories.

Further improvements in the construction of the instrument are suggested, which may increase its accuracy and broaden the permissible range of substrate concentration.

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ENZYME ASSAY WITH A COAXIAL VISCOSIMETER

II. Method for the Assay of the Amyloclastic Activity of Pancreatin

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VARIOUS authors[1-3] have shown the validity of assaying amyloclastic activity with Ostwald viscosimeters. In the research laboratory this method of assay is generally simpler and more rapid than chemical techniques. However, Elman[4] advised against its use in clinical laboratories. Voegtlin[5] has described a rugged coaxial viscosimeter and given several *a priori* reasons why a coaxial viscosimeter is preferable to Ostwald's capillary type in measuring tryptic and amyloclastic activity. In the present study, a substrate and technique for assay of amylase activity, suitable for clinical or commercial laboratory use, has been developed for Voegtlin's instrument. The results are compared with our modification of the Somogyi copper-reduction test.

METHOD

Substrate.—One-pound lots of rice starch (Coleman & Bell) are heated in an open container at 90° C. for forty-eight hours and stored at 45 to 50° C. Three per cent starch paste is prepared from the dried powder as follows: Heat 190 ml. of distilled water almost to the boiling point. Prepare a smooth paste of 9.0 Gm. dried rice starch and 40.0 ml. cold distilled water. Add paste to hot water, stirring constantly. Rinse paste container with 40.0 ml. cold water, and add rinsings to hot paste. Stir and cover. Boil gently for 5 minutes. Remove from heat and add 30.0 ml. of 1/15 M phosphate buffer, pH 7.15. Immediately pour substrate into a flask and stopper tightly. *Do not agitate.* Cool to below 37.5° C., but do not cool or store below 20° C. Lumpy substrates are unsatisfactory, but the small amount of scum that may form on the surface of the substrate can be avoided in taking samples. The substrate should be pH 7.15, and should not drop below 6.8 during digestion. Some lots of starch powder may require larger amounts of buffer. Although fresh substrates were used during standardization of the test, twenty-four to forty-eight hour old ones, which had been stored at room temperature and showed no marked decrease in viscosity, were satisfactory when restandardized daily.

Standard Pancreatin Solutions.—Suspend 250 mg. ± 0.1 mg. U.S.P. pancreatin (Parke, Davis & Co., Lot H2411D was used) in 25.0 ml. sterile 0.85 per cent sodium chloride solution. Centrifuge exactly 10 minutes at 2,000 r.p.m., and collect the supernate. Occasionally, the supernate must be recentrifuged for two to three minutes to remove all visible particles. From this 1 per cent extract, prepare suitable dilutions in saline solution. Store dilutions and extract in ice bath until discarded. Normally, these solutions were stable over a period of eight hours. However, accidental contamination with alkali (from soap deposits on glassware, etc.) caused marked loss of activity within one hour. Other dehydrated substances containing amylase were extracted in a similar manner, usually at 1 per cent concentration.

Testing.—With the oil bath of the viscosimeter operating at 37.5° C. and the pendulum in position, place 7.5 ml. of substrate in the cup. Start the motor and run it until a constant reading is obtained (two to three minutes). Without stopping the motor, add exactly 0.5 ml. of the enzyme sample. Start timing the reaction as the last of the enzyme is added.

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Record as the end point of the test the time in tenths of a minute at which the scale reading indicates an 80 per cent decrease in the viscosity of the substrate. When the end point is reached in less than three minutes, the enzyme should be further diluted. Tests taking longer than twenty minutes may be affected by evaporation and should be avoided.

Controls.—Substrate stability is controlled by making a run (1) with substrate alone equivalent to the longest anticipated run, and (2) with 0.5 ml. of saline solution or boiled enzyme solution. After temperature equilibration has occurred, no change in the viscosity of the substrate should be noted except that caused by simple dilution when the inactive sample is added.

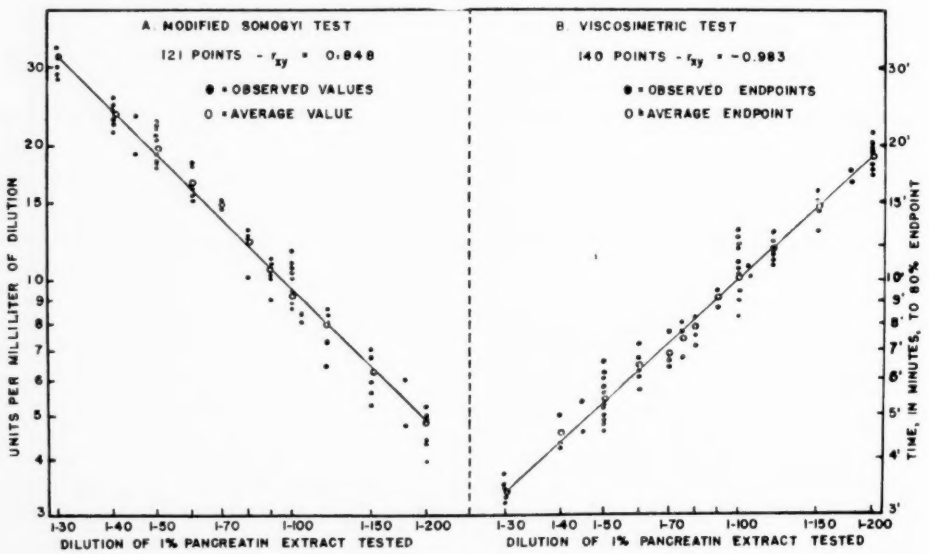
A. MODIFIED SOMOGYI TEST
121 POINTS - r_{xy} = 0.848
● = OBSERVED VALUES
○ = AVERAGE VALUE
UNITS PER MILLILITER OF DILUTION
DILUTION OF 1% PANCREATIN EXTRACT TESTED
B. VISCOSIMETRIC TEST
140 POINTS - r_{xy} = -0.983
● = OBSERVED ENDPOINTS
○ = AVERAGE ENDPOINT
TIME, IN MINUTES, TO 80% ENDPOINT
DILUTION OF 1% PANCREATIN EXTRACT TESTED

Fig. 1.—Results of modified Somogyi tests (*A*) and of viscosimetric tests (*B*), plotted against dilution of 1 per cent pancreatin extract tested. (A few dilutions were tested with two viscosimetric substrates, resulting in two points per dilution.)

STANDARDIZATION OF THE TEST

The coaxial viscosimeter test was standardized against the modified Somogyi method* by performing parallel tests with the same dilutions of standard pancreatin extracts. Fig. 1 shows that the straight-line relationship between enzyme concentration and time to viscosimetric end point is similar to that between concentration and glucose production in the modified Somogyi test. The coefficient of correlation between known enzyme concentration and observed value for the modified Somogyi test was 0.85, and between concentration and the reciprocal of time to end point was 0.98 for the viscosimetric test.

The causes of variability in both tests were sought. The activity of the reference lot of pancreatin, according to modified Somogyi tests on 204 dilutions of 46 lots of 1 per cent pancreatin extract, was 96.55 units per milligram,

*The original Somogyi test[6] was not applicable to pancreatin extracts of duodenal fluid. Our modification consists of replacing the acid-saline solution by equal parts 1/15 M phosphate buffer, pH 7.15, and 0.85 per cent NaCl solution. The buffer and starch paste are warmed to 40° C. separately and mixed immediately before adding the substrate to the sample. Where necessary, the total volume of the test was adjusted to provide sufficient starch for maximal glucose production.[7]

with a standard error of ± 0.63 and a coefficient of variation of ± 9.32 per cent. When replicate tests were performed with one dilution of extract and one lot of substrate, the results of viscosimetric tests varied by ±2 to ±4 per cent and those of modified Somogyi tests by ±4 to ±7 per cent. If dilutions of several lots of pancreatin extract were tested with one lot of substrate, the variability of each test increased by about ±1 per cent. It was concluded that any variations in lots of standard pancreatin extract used as controls in the viscosimetric test would be unimportant.

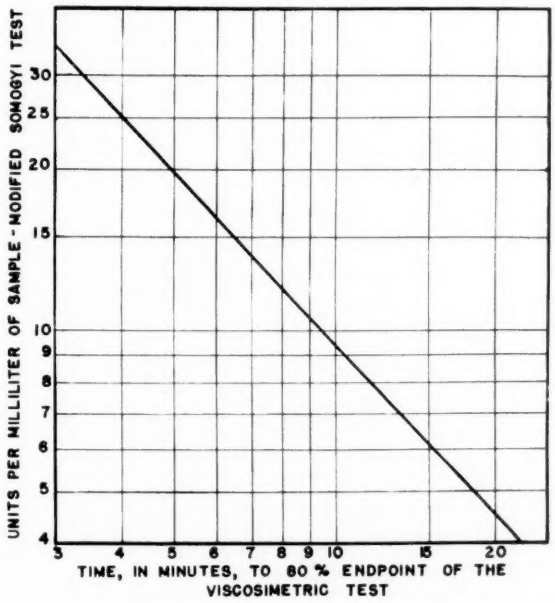

Fig. 2.—Standard calibration curve. Line: Log. Y = 2.03284-1.05575 Log. X; r_{xy} = 0.89; P_{xy} = 0.99.

The variability of the modified Somogyi tests used in calculating the activity of the reference lot of pancreatin was directly related to variations in the reactivity of the starch paste between lots, and as individual lots aged. No mention of this source of error in the Somogyi test was found, but the necessity for daily checks of viscosimetric substrates has been recognized.[1-3] As indicated earlier, the viscosity of substrate lots used in the coaxial viscosimeter varied. The more viscous the substrate, the more rapidly the 80 per cent end point was reached.

In calculating the curve in Fig. 2, tests on substrates with a given viscosity could be used. However, the viscosity of substrate lots varied frequently enough to require correction. Two methods were compared. The first was to construct a calibration curve for each lot of substrate by plotting the end points obtained from tests of three to five dilutions of standard pancreatin extract against the activity of each dilution when 95.0 units per milligram was used as a working value for the standard pancreatin. A curve based on four dilutions of extract

TABLE I. SUMMARY OF TESTS ON SAMPLES OF CRUDE PANCREATIN

LOT NO.	NO. OF TESTS*		MEAN VALUE (UNITS/MG.)				COEFFICIENT OF VARIATION (%)			
				VISCOSIMETRIC TEST				VISCOSIMETRIC TEST		
				FROM STANDARD CURVE				FROM STANDARD CURVE		
	SOMOGYI	VISCOSIMETRIC	MODIFIED SOMOGYI TEST	DIRECT READING	INTERPOLATED READING	FROM DAILY CURVE	MODIFIED SOMOGYI TEST	DIRECT READING	INTERPOLATED READING	FROM DAILY CURVE
1512	10	14	12.26 ± 0.47	10.49 ± 0.23	11.85 ± 0.24	11.76 ± 0.34	12.1	8.2	7.5	7.7
3392	8	12	18.97 ± 0.60	16.15 ± 0.62	17.43 ± 0.49	17.63 ± 0.42	8.9	12.1	9.0	8.3
1651	10	16	102.1 ± 3.5	86.92 ± 2.69	98.33 ± 2.16	98.74 ± 2.33	10.8	12.4	8.8	9.5
3586	12	16	74.12 ± 2.25	67.59 ± 2.67	76.11 ± 1.29	77.95 ± 0.80	10.5	15.8	6.8	4.1
3400	12	16	207.3 ± 4.9	186.9 ± 8.6	214.9 ± 3.6	219.1 ± 4.0	8.2	18.2	6.8	7.3
3580	12	16	214.3 ± 6.1	198.4 ± 8.0	225.6 ± 4.0	230.3 ± 4.6	9.8	16.1	7.1	8.0

*On some days two viscosimetric substrates were used, and treated as two tests in analyzing results.

could be established in thirty-five to forty-five minutes. The second, and more rapid, method utilized the standard calibration curve in Fig. 2. Each substrate was tested with two dilutions of standard pancreatin extract which gave end points within three to seven minutes. From each end point a factor was calculated as follows:

$$\frac{\text{Calculated end point at given dilution of standard enzyme (from graph)}}{\text{Observed end point at same dilution of standard enzyme}}$$

The factors were averaged and the observed end point for the test on unknown material was multiplied by the average factor. This product was read on the standard calibration curve as activity per milliliter of material tested.

Table I shows the results of tests on a group of crude pancreatin samples read on the standard calibration curve (1) without and (2) with correction for substrate variability, and (3) on daily calibration curves. When compared with the results of modified Somogyi tests on the same samples, method 1 was unsatisfactory, although it differentiated between the first five samples which were uniform according to U.S.P. tests. The results of methods 2 and 3, both more consistent than the chemical tests, were so similar that a daily calibration curve seems unnecessary.

DISCUSSION

When pancreatin extracts were tested with one lot of substrate, the accuracy of tests with the coaxial viscosimeter was equal to or greater than that of modified Somogyi tests performed under the same conditions. While the accuracy of the original Somogyi test when one sample is tested with several substrates has not been reported, the accuracy of the modified test was decreased under these conditions. Allowance for differences in substrate lots in interpreting viscosimetric tests on unknown samples of pancreatin made these tests more accurate than chemical tests on the same samples.

Assaying of amyloclastic activity with the coaxial viscosimeter has proved rapid and simple, as well as accurate. In making a large number of tests, the Somogyi test decreased in accuracy if more than two or three tubes were incubated at one time. If the activity range of samples was not accurately guessed, time was lost in repeating part or all of the chemical test. Huggins and Russell[8] developed a colorimetric test comparable in accuracy to the Somogyi test, but used a longer incubation period. Clark's[9] rapid colorimetric test was designed for use in surgical emergencies and is intended to be only roughly quantitative. The viscosimetric and colorimetric methods of assay were not compared, but others[10] find that it is often necessary to repeat the Huggins-Russell test to hit the range of highly active substances such as pancreatic fluid.

Preliminary tests with the coaxial viscosimeter indicated that it can be used in assaying blood serum and other body fluids.

SUMMARY

A method for using the coaxial viscosimeter to assay the amyloclastic activity of pancreatins has been described and compared with a modified Somogyi test.

The method is simpler and more rapid than the Somogyi test.

In tests on unknown samples of pancreatin, the method was less variable than the modified Somogyi test.

An important variable in both tests was substrate variability and, while it can be allowed for in interpreting viscosimetric tests, improvements in the substrates are desirable.

The technical assistance of Joseph W. Voegtlin, Harrower Fellow 1948-1950, and of David F. Wolter was important in the development of this test.

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APPARATUS FOR THE RAPID ACCOMPLISHMENT OF TURBIDIMETRIC OR COLORIMETRIC MEASUREMENTS

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THE use of microbiological or biochemical assays requiring numerous colorimetric or turbidimetric readings is increasing. In making these measurements, the filling and discarding of material from the sample tube or cuvette is a major time-consuming operation. A simple apparatus has been devised that permits the rapid filling, emptying, rinsing, and draining of a cuvette without its removal from the colorimeter. No structural modification of the colorimeter is required, and only common laboratory materials are needed. The apparatus has been used successfully with a Klett-Summerson photoelectric colorimeter (Model 1834) and a Lumetron photoelectric colorimeter (Model 402-E), and appears to be adaptable to any machine which employs a test tube for a cuvette. In its essentials, the apparatus consists of a wide-stemmed funnel attached to the top of the cuvette and a rigid suction tube which can be dropped through the funnel to the bottom of the cuvette but which is adjusted so that it will just fail to touch the bottom. The rigid suction tube is connected by means of flexible tubing to a collecting bottle which, in turn, is attached to a vacuum source. If noninfectious, sink-disposable material is being measured, the collecting bottle may be omitted and a water aspirator is a satisfactory source of vacuum.

The apparatus (Fig. 1) designed specifically for the Klett-Summerson photoelectric colorimeter is described in detail below. The cuvette (*2*)* used measures 97 mm. by 12.5 mm. A powder funnel (*1*) having a diameter of 80 mm. and a stem diameter of 16 mm. is attached to the top of the cuvette by a 30 mm. piece of ⅜ inch rubber tubing (*3*). These parts are "short coupled" so that there is maximum rigidity of the assembled unit. The trade mark on the funnel is aligned with the positioning spot on the cuvette to serve as a positioning indicator. The suction tube (*5*) is a piece of fire-polished glass tubing (outer diameter, 5 mm.; length, 21 cm.). The tube is inserted through a snugly fitting hole in a No. 24 rubber stopper (*4*) so that when the suction tube is dropped through the funnel into the cuvette the stopper is inverted. The position of the stopper on the suction tube is adjusted so that when the suction tube is in the cuvette and the stopper is resting in the funnel, the tip of the suction tube clears the bottom of the cuvette by approximately 0.5 mm. In order to prevent scratching of the cuvette a piece of 3 mm. rubber tubing is slipped over the suction tube almost up to the rubber stopper. This tubing is cut off exactly even with the lower end of the suction tube. Three notches about 5 mm. deep are cut equally spaced around the largest circumference of the rubber stopper. The suction tube assembly then is connected with 3 mm. rubber tubing (*6*) of convenient length to a collecting bottle having the conventional two-holed rubber stopper with long and short pieces of glass tubing. The short tube of the collecting bottle is connected to a source of moderate vacuum.

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*All numbers in parentheses refer to Fig. 1.

In operation, the vacuum is adjusted to induce a mild suction. The first sample to be measured is poured into the cuvette. Bubbling can be minimized by pouring the sample onto the inner surface of the funnel and permitting it to flow down the sides of the cuvette. The desired light transmission measurement then is made, and, when this is completed, the suction tube is dropped through the funnel to draw the sample over into the collection bottle. With the suction tube still in place, distilled water or other wash solution from a wash bottle is squirted into the funnel. This passes through the notches in the rubber stopper, flows evenly down the sides of the cuvette, and is drawn off as it reaches the bottom. If the length of the suction tube is properly adjusted, the drainings from the sidewalls are removed and little, if any, visible

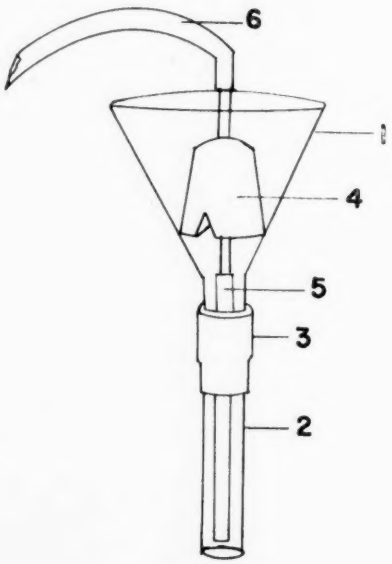

Fig. 1.

liquid remains in the cuvette. The suction tube is then removed from the cuvette and the apparatus is ready to receive the next sample. In practice, it has been found that the sample removal is sufficiently complete that when replicates or other similar samples are being measured, the washing step may be omitted without introducing serious error.

Through the use of this apparatus, it has been found possible to double or triple the over-all rate with which turbidimetric readings can be made. Direct comparisons with the results obtained by the conventional method indicate that there is no sacrifice of accuracy. Moreover, several troublesome sources of occasional error such as scratches, faulty tube positioning, and finger marks have been practically eliminated. If infectious material is being studied, the whole suction assembly may be autoclaved after use without disassemblying in any way. The cuvette assembly may be chemically disinfected or autoclaved, depending on the construction of the cuvette itself.